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Injury induced plasticity in primary neuronal culture and the mature brain

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SUMMARY

The mature central nervous system (CNS) is unable to fully repair after traumatic brain injury (TBI). Following an injury to the adult brain there is a complex sequence of events that take place across a broad time course. The cellular mechanisms evoked by the brain when attempting to respond to injury need to be fully elucidated in order to devise effective therapeutic interventions. The current thesis is based upon the hypotheses that recovery following trauma will require the activation of reactive and compensatory plasticity and that the mechanisms underlying this plasticity are intrinsically different for excitatory and inhibitory cortical networks.

This thesis investigated the reactive and regenerative alterations associated with the neuronal response to structural injury. Studies focused on the potential for plasticity following injury, specifically comparing the post-injury characteristics of excitatory and inhibitory neurons. It demonstrated that the neuronal response to injury was subclass specific given that structural injury *in vitro* induced an axonal regenerative response in excitatory neurons and significant dendritic remodeling in a subpopulation of interneurons. Additionally, results from functional studies indicated that these two mechanisms could both be contributing to the development of post-injury hyperexcitability.

This thesis further investigated the interneuron response to injury using a clinically relevant *in vivo* model of diffuse and focal injury, the lateral fluid percussion injury model. Immunohistochemistry confirmed that interneurons were not lost after mild injury but, consistent with *in vitro* data, underwent subpopulation specific morphological alterations. Furthermore, electrophysiological studies *in vivo* demonstrated changes in inhibitory transmission that could lead to overall changes in excitatory/inhibitory balance as demonstrated in *in vitro* studies.

Finally this thesis investigated a potential drug treatment for traumatic injury. Neuronal cytoskeletal alterations, in particular the loss and misalignment of microtubules, are considered a hallmark feature of the degeneration that occurs after TBI. Therefore, microtubule-stabilizing drugs are attractive as potential therapeutics for use following TBI. This thesis characterized the effect of the brain penetrant microtubule-stabilizing agent Epothilone D (Epo D) on post-injury axonal sprouting in an *in vitro* model of CNS trauma. Epo D was found to modulate axonal sprout

number in a dose dependent manner, increasing the number of axonal sprouts generated post-injury. Specific effects on excitatory neurons were also found. This thesis demonstrated that Epo D significantly increases the neuronal regenerative response following structural injury.

In summary this thesis demonstrated intrinsic differences in how excitatory neurons and inhibitory interneurons respond to injury. Moreover, it established a possible link between this differential response and alterations in excitatory/inhibitory balance in the cortex after injury. Furthermore, it identified a possible therapeutic intervention for enhancing regeneration following CNS trauma.

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ABBREVIATIONS

AMP	adenosine monophosphate
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
CGE	caudal ganglionic eminence
CB	calbindin D-28
CCK	cholecystokinin
CO_2	carbon dioxide
°C	degrees Celsius
CR	Calretinin
CNS	central nervous system
DAI	diffuse axonal injury
DIV	days *in vitro*
FS	fast spiking
FPI	fluid percussion injury
GABA	gamma-Aminobutyric acid
GAD	glutamate decarboxylase
GAP-43	growth-associated protein 43
GDNF	glial derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPI	glycosyl phosphatidyl-inositol
IS	irregular spiking
HBSS	Hanks Buffered Salt Solution
LGE	lateral ganglionic eminence
IgG	immunoglobulin G
IgM	immunoglobulin M
l	litre

LS	late spiking
µl	microlitre
µm	micrometre
µM	micromolar
mm	millimetre
MAG	myelin associate glycoprotein
MAP-2	microtubule-associated protein 2
MGE	medial ganglionic eminence
mV	millivolt
NPY	neuropeptide Y
OMgP	oligodendrocyte myelin glycoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
PNS	peripheral nervous system
POA	embryonic preoptic area
PTEN	phosphatase and tensin homolog
PV	parvalbumin
RMP	resting membrane potential
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SOM	somatostatin
SVZ	subventricular zone
TBI	traumatic brain injury
TBS	tris buffered saline
TTBS	tris buffered saline with tween
TG	transgenic mouse
VIP	vasoactive intestinal peptide

1. INTRODUCTION

Traumatic brain injury (TBI) is defined as an injury to the brain that is the consequence of an external mechanical force and alters normal cognitive, physical and/or emotional functioning (Fortune and Wen, 1999, Blennow et al., 2012, Sullivan et al., 2016). Epidemiological studies indicate that the worldwide incidence of TBI has risen in recent years (Rusnak, 2013, Dewan et al., 2016). This is principally due to the increased number of motor vehicle accidents in low to middle income countries, the increased incidence of falls among the elderly in high income countries and the global increase of military-related traumatic injuries (Langlois et al., 2006, Maas et al., 2008, McKee at al., 2014). According to the 'Centre for Disease Control and Prevention' in the US there are around 1.7 million people that sustain a TBI in that country each year, mainly young adults, and this results in approximately 52,000 deaths per year (Malpass, 2013). The high incidence of TBI among young people is particularly devastating as it frequently results in prolonged or life-long motor and/or cognitive impairments and places a complex socio-economic and medical burden upon society (Roozenbeek et al., 2013). In Australia the most common causes of TBI include motor vehicle accidents, falls, injuries derived from violence and sport-related injuries. In accordance with the last report of 'The Victorian Neurotrauma Initiative' the current cost burden of new cases of TBI in the country is estimated to be around 8.6 billion dollars per year (The Victorian Neurotrauma Initiative, 2010-2011).

Even though TBI continues to be a leading cause of death and disability worldwide, the cellular mechanisms that underpin the central nervous system (CNS) degeneration and its inability to induce cellular regeneration after injury are not yet understood. As a result it is not surprising that there are no therapeutic interventions that can minimize or reverse the primary damage and prevent the complex secondary pathologies that develop following injury to the adult brain (Bruns and Hauser, 2003). Consequently, determining the basis for the inability of the mature CNS to regenerate after injury, documenting the specific functional alterations in the injured brain, and identifying therapeutic targets for the development of appropriate interventions for the treatment of TBI remain important and relevant avenues for research.

The heterogeneity of TBI is still considered one of the major barriers to finding effective therapeutic interventions. However, significant headway has been made in

recent years with regards to characterizing the types of injury and trying to link specific patterns of damage to appropriate therapies.

1.1. CLASSIFICATION AND TYPES OF BRAIN INJURY

There are two distinct categories of injuries that can lead to TBI; contact and non-contact injuries (reviewed in Finnie, 2002). Contact injuries are a consequence of a direct impact to the head and can be further classified as penetrative or not penetrative, according to the presence or absence of scalp laceration or skull fracture. Non-contact injuries are not penetrative and are produced as a result of an abrupt acceleration or deceleration of the head that results in a rotation of the brain within the skull (Davis, 2000, Werner and Engelhard, 2007). Both contact and non-contact injuries can result in focal or diffuse damage to the brain (Povlishock and Katz, 2005). Focal damage is usually the result of haemorrhagic lesions within the gray matter, or at the gray-white matter interface which usually induce ischemia and neuronal death by necrosis or apoptosis (Raghupathi, 2004). Diffuse injuries usually occur as a consequence of the differential movement of the brain and the cranium, and produce widespread brain dysfunction with little or no macroscopic damage but important microscopic axonal changes (DeKosky et al., 2013). These changes are usually characterized by the presence of swellings or varicosities along the length of axons and the presence of large terminal bulbs known as retraction bulbs (Povlishock and Christman, 1995, Smith et al., 2003a, Johnson et al., 2013, reviewed in Siedler et al., 2014).

The brain damage observed after a TBI can be further classified as primary or secondary damage. Primary damage involves any damage to the brain that is the direct result of an initial injury and occurs within seconds to minutes. The alterations produced by the primary damage can be observed at a macroscopic or a cellular level and include neural disruptions, shearing of white matter tracts, focal contusions, haematomas and diffuse swelling (Maas et al., 2008). Primary injuries often trigger a series of secondary injury mechanisms. Secondary injuries involve a complex sequence of events across a broad time course and influence different regions of the brain. They consist of secondary cellular processes and biochemical cascades that are developed over hours, days or even weeks after the initial trauma and dramatically worsen the damage caused by the primary injury (Graham et al., 2000). These secondary injury processes include: ischemia caused by decreased cerebral blood flow; brain swelling; excitotoxicity due to release of excitatory neurotransmitters; free-radical generation; calcium-mediated damage, oedema and mitochondrial

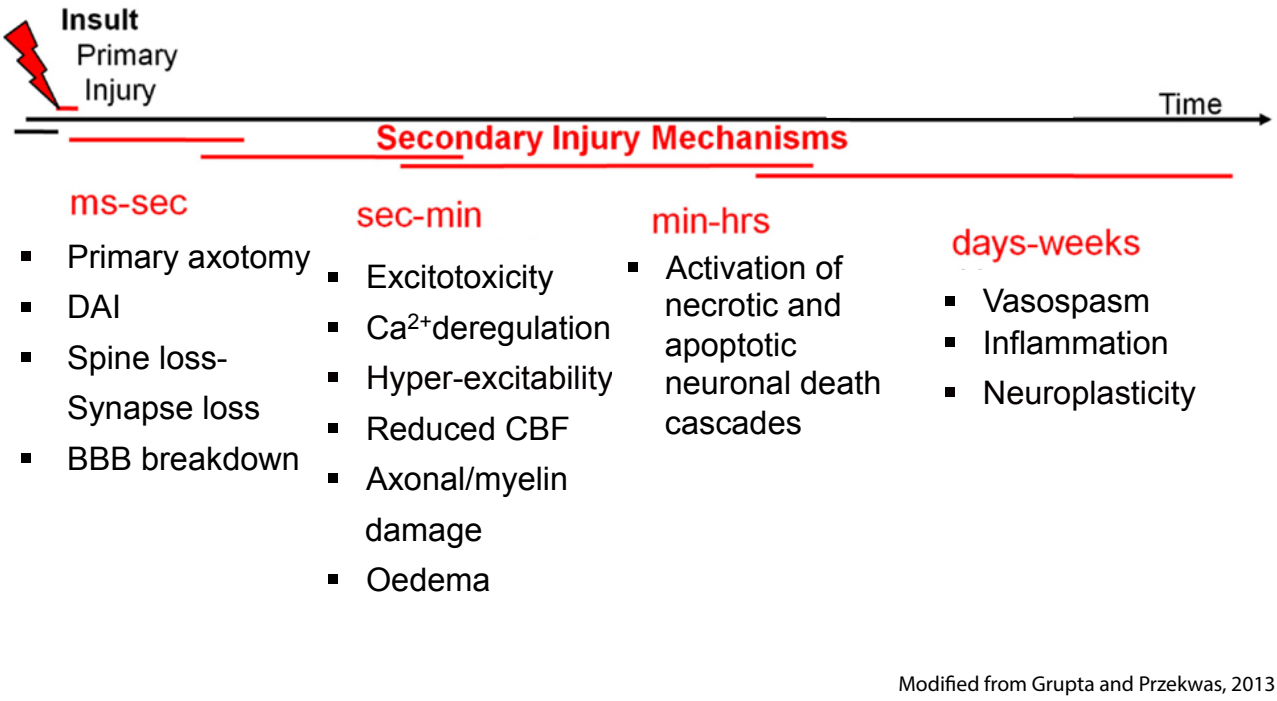
dysfunction that generates energy failure (Gupta and Przekwas, 2013) (Fig 1.1). Furthermore, there is the initiation of necrotic and apoptotic neuronal cell death cascades that result in neuronal degeneration and finally denervation (Maas et al., 2008, Buki and Povlishock, 2006, Saatman et al., 2008). Brain oedema in particular is a common consequence of TBI and the its development is one of the most significant predictors of outcome after brain injury (Donkin and Vink, 2010). There are two main types of oedema, cytotoxic and vasogenic brain edema. Cytotoxic edema is related with a failure in ATP-dependent Na+/K+-pumps during energy shortage, increase in water content within the intracellular compartment as a result of an osmotic gradient and cell swelling. Vasogenic edema occurs when the blood–brain barrier (BBB) becomes leaky allowing an influx of plasma from the vasculature into the extracellular space (Borgnia et al., 1999, Unterberg et al., 2004, Michinaga et al., 2015).

In a clinical setting traumatic brain injuries can be further classificed according to clinicopathological parameters in a severity spectrum as mild, moderate and severe injuries. The severity of a brain injury is usually evaluated using the 15 point Glasgow Coma Scale (GCS), which takes into consideration 3 major parameters: verbal, motor, and eye-opening reactions to stimuli. In mild TBI, the GCS score is ≥13, in moderate TBI it is 9–12, and in severe TBI it is ≤8. Importantly, the same injury severity may present different pathological and clinical outcomes due to the complexity of secondary injury mechanisms that are activated after injury (Fig 1.1) (Pitkanen and Immonen, 2014).

After an injury to the brain, the cortex is particularly susceptible even after a mild impact due to its complex and highly organized structure.

1.2. THE NEOCORTEX

The mammalian cerebrum is comprised of two hemispheres that are 'coated' by the neocortex. The neocortex is a thin, extended sheet of tissue that contains up to $28x10^9$ neurons in humans and approximately $5x10^6$ neurons in mice and is responsible for higher order brain functions (Kaas, 1993, Mountcastle, 1997). The mammalian neocortex consists of two neuronal types, broadly categorized based on morphology: pyramidal neurons or principal cells, and non-pyramidal neurons or interneurons (Parnavelas, 2000, Molnar and Cheung, 2006). The structure of the neocortex in humans and mice is relatively uniform consisting of six horizontal layers segregated principally by neuronal cell type, size and density, denoted layer I



Modified from Grupta and Przekwas, 2013

Figure 1.1. Primary and secondary injury mechanisms.

Schematic representation of post-injury mechanisms activated after an initial traumatic injury to the brain.

through VI from the most superficial to deep layer (Fig 1.2). The molecular layer, layer I, is a relatively cell free layer. Layers II and IV, the external and internal granular layers are comprised of relatively small locally projecting interneurons. Layers III and V contain characteristic large cortical pyramidal neurons and are designated the external and internal pyramidal layers, respectively (Douglas and Martin, 2004). Interactions between cortical pyramidal neurons and interneurons facilitate highly integrated cortical processing and underlie all coordinated neuronal activity (Lehmann et al., 2012, Kubota, 2014).

1.2.1. Corticogenesis

The development of the cortex, termed corticogenesis, is a highly dynamic and complex process involving tightly regulated patterns of neuronal proliferation and migration. Neurons destined to form the neocortex are generated at the rostra-dorsal region of the neural tube, the telencephalic pallium (Parnavelas, 2002, Noctor et al., 2004). The two distinct groups of neurons in the neocortex, glutamatergic pyramidal neurons and GABAergic interneurons, originate from different regions of the telencephalon (Anderson et al., 1997, Anderson et al., 2002). Pyramidal neurons are generated in the germinal ventricular zone of the dorsal telencephalon. Interneurons, on the other hand, are generated from neural stem cells located in the ventricular zones of the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and caudal ganglionic eminence (CGE) of the ventral telencephalon (Parnavelas, 2000, Wichterle et al., 2001, Nery et al., 2002, Parnavelas, 2002, Rudy et al., 2011, Welagen and Anderson, 2011). Pyramidal neurons and interneurons also differ in the mode of migration that new neurons use to reach their destination in the cerebral cortex. Cells destined to become pyramidal neurons use radial migration, guided by radial glia, whereas interneuron precursors use tangential migration to reach the developing cortex (Mountcastle, 1997, Nadarajah et al., 2003, Kriegstein and Noctor, 2004).

A number of studies have demostrated that different areas within the ventral telencephalon are involved in the generation of specific interneuron subpopulations. It has been suggested that the distinct origins of cortical interneuron subtypes may account for their heterogeneity in the cortex (Anderson et al., 2001, Anderson et al., 2002, Fogarty et al., 2007, Kepecs and Fishell, 2014).

The MGE is the principal source of cortical interneurons in rodents during early stages of telencephalic development (E11.5-E14.5) and is the origin of parvalbumin

(PV) and somatostatin (SOM)-expressing interneurons (Wichterle et al., 2001, Xu et al., 2003, Xu et al., 2004, Butt et al., 2005, Miyoshi et al., 2007). After the MGE, the CGE is the next most significant sorce of cortical interneurons, producing around 15-30 percent of all interneurons (Lee et al., 2010, Miyoshi et al., 2010). The CGE produces mainly bipolar Calretinin (CR) expressing interneurons but mostly during later stages of neurogenesis (E14.5-E16.5). It also produces vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY)-expressing cells (Xu et al., 2004, Welagen and Anderson, 2011). The CGE is continuous with, and shares many molecular properties with, the LGE, which gives rise to a small fraction of cortical interneurons that do not include SOM- or PV-expressing cells (Ma et al., 2012).

Another area that generates cortical and hippocampal interneurons is the embrionic preoptic area (POA). This area gives rise to a relatively homogenous population of neurons found in the superficial layers of the cortex that exhibit a complex, multipolar morphology. Most neurons arising from the POA are NPY positive and SOM negative (Gelman et al., 2009).

1.2.2. Neocortical neurons

1.2.2.1. Pyramidal neurons

Pyramidal neurons are the primary glutamatergic excitatory cells of the neocortex (Elston, 2003, Fishell and Hanashima, 2008). They have large cell bodies, ranging from 10-50 μm in diameter, one long projecting axon and extensive dendritic arborisations that have specialized synaptic protrusions termed dendritic spines (Garcia-Lopez et al., 2006). They constitute around 60 to 70 percent of the neurons in the cortex, and represent a morphologically homogeneous population despite having variable synaptic connections and physiological characteristics related to their intrinsic membrane properties (Zhang, 2004). Differences in the type and distribution of ion channels correlates with differences in membrane properties, the shape of action potentials, and the way in which they transform synaptic input into spike output. Pyramidal neurons can therefore be classified by their response to current injections, as regular spiking neurons and burst neurons. Most pyramidal neurons in layers II-VI respond to sustained depolarization by producing a train of action potentials at a regular frequency that exhibit spike-frequency adaptation (accommodation). By contrast, pyramidal neurons in layer V respond to sustained depolarization by firing one or more bursts of action potentials, and have been

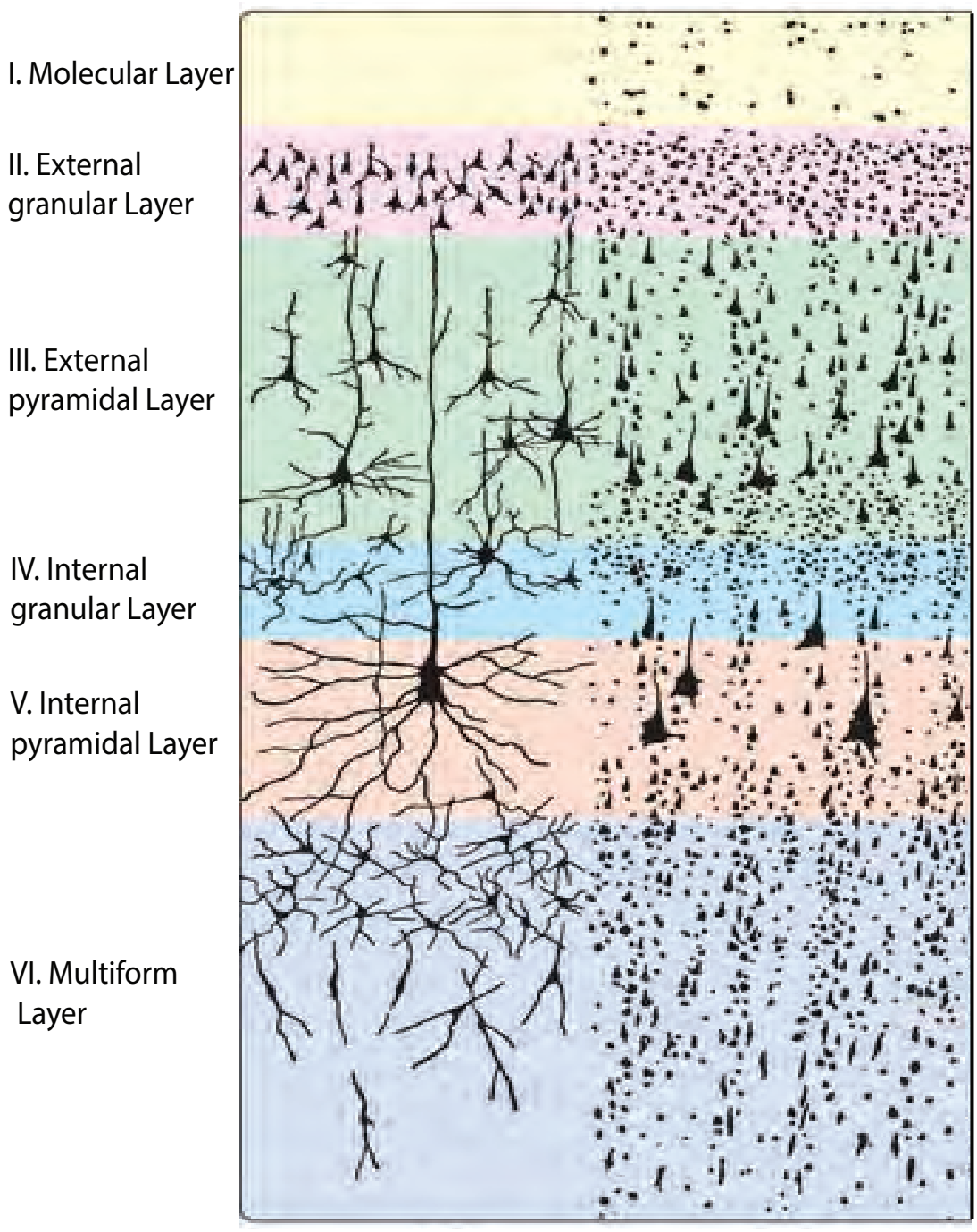


Figure 1.2. Layers in the cerebral cortex.

Representation of the six layers of the cortex. The name of each layer reflects the cell types that predominantly populate that layer (Adapted from Nolte, 2009).

classified as weak or strong bursting depending on the number of spikes they generate (Williams and Stuart, 1999).

Pyramidal neurons have been further classified according to their projection targets as Intratelencephalic type (IT type) that project within the telencephalon or Pyramidal type (PT-type) that project beyond the telencephalon. It has been described that PT-type cells have narrower action potentials with more rapid repolarization, whereas IT-type cells have broader action potentials with slower repolarization (Suter et al. 2013; Shepherd, 2013, Guan et al. 2015). Furthermore in the last couple of years evidence has emerged indicating that genetically-defined subpopulations of neocortical pyramidal neurons (etv1 and glt) also differ in action potential properties (Pathak et al. 2016, Cembrowski et al, 2016).

1.2.2.2. Interneurons

Interneurons are a group of non-spiny or sparsely spiny neurons that are the major source of inhibition in the neocortex. Their axons are restricted to the same anatomical region as their cell bodies and dendrites (Letinic et al., 2002), and they comprise around 20-30 percent of all neurons in the neocortex (Defelipe, 2002). Interneurons release gamma-Aminobutyric acid (GABA) as a neurotransmitter and have been functionally implicated in the regulation of synaptic integration, the probability and temporal dynamics of action potential generation and the plasticity of pyramidal cells (Huang et al., 2007, Kepecs and Fishell, 2014).

Unlike the more homogenous pyramidal populations, interneurons are very diverse in their morphology and functionality in terms of their intrinsic membrane properties, connectivity and dynamics of input and output synapses (Buzsaki et al., 2004, Caputi et al., 2013) (Fig 1.3). Their complexity and diversity has made it difficult to achieve a systematic method of sub-type classification (DeFelipe et al., 2013). Currently the methods used for classifying inhibitory interneurons are based on morphological features in combination with neurochemical and electrophysiological characteristic (reviewed in (Druga, 2009). The expression of the calcium-binding proteins PV, CR and calbindin-D28K (CB) has been used widely and has proven instrumental in categorizing subtypes of interneurons. According to the synaptic connections they make with pyramidal cells they can be classified as soma and proximal dendrite targeting cells, dendrite targeting cells, axon targeting cells and dendrite and tuft targeting cells. (Buzsaki et al., 2004).

Soma and proximal dendrite targeting interneurons

Soma and proximal dendrite targeting inhibitory interneurons can be further classified as large-, small- and nest-basket cells according to their axonal and dendritic morphology, expression of calcium binding proteins and neuropeptides (Wang et al., 2002). Basket cells are the most common type of interneuron in the neocortex representing around 50% of all inhibitory cells. They target the soma and proximal dendrites of pyramidal cells (Freund, 2003, Druga, 2009). Large Basket cells have long multipolar dendrites and extensive axonal arborizations. They represent the major source of lateral inhibition across columns within the cortical layer containing their cell bodies, but also inhibit cells within the upper and lower layers. Large basket cells usually express the calcium binding proteins CB and PV and the neuropeptides, neuropeptide Y (NPY) and cholecystokinin (CCK) (Rudy et al., 2011). They also occasionally express somatostatin (SOM) and CR, but never vasoactive intestinal peptide (VIP) (reviewed in Markram et al., 2004, Druga, 2009). Small basket cells have dense and very complex axonal arborizations and rarely elaborate axons beyond the cortical layer or column in which they reside. Small basket cells are usually present as multipolar cells in layer IV and as bitufted or bipolar cells in layer II/III. They express the same calcium binding proteins and neuropeptides as large basket cells, but they also express VIP (Markram et al., 2004). Nest basket cells are similar to large and small basket cells, but can be identified by their bird's nest-like morphology and the their lack of CR and VIP expression (Wang et al., 2002).

Dendrite targeting interneurons

The sub-population of interneurons that synapse onto dendrites are morphologically and functionally more diverse than any other sub-group, and include double bouquet cells, bipolar cells, neurogliaform cells and bitufted cells. Double bouquet cells usually have a bitufted dendritic morphology and a characteristic thick axon that resembles a horsetail. They are distributed across all layers but are more common in supragranular layers (Yanez et al., 2005).Double bouquet cells are a source of interlayer, and probably intracolumnal inhibition. They express CR and CB and can also express VIP or CCK but not PV, SOM or NPY (Markram et al., 2004, Druga, 2009). Bipolar cells are small cells with ovoid soma and narrow bipolar dendrites that extend vertically to layer I and down to layer VI (DeFelipe, 1997). They are CR positive and can express VIP (Cauli et al., 2014). Neurogliaform cells are

small interneurons with a highly branched, dense dendritic field that forms a sphere and also elaborates a thin but densely branched axon. A subset of neurogliaform cells co-express CB and NPY. Bitufted cells are similar to bipolar and double bouquet cells. They usually have an ovoid soma from where two primary dendrites project in opposite directions to each other. They are found in layers II-VI and express CB, CR, NPY, VIP, SOM and CCK, but not PV (Druga, 2009).

Dendrite and tuft targeting interneurons

The principal dendrite and tuft targeting interneuron is the Martinotti cell. Martinotti cells represent about 15 percent of the total interneuron population. They are found in layers II–VI, and project their axons toward layer I where they inhibit the tuft dendrites of pyramidal neurons. Their axons can also project horizontally to inhibit cells in other columns, providing cross-columnar inhibition (Wang et al., 2004). They have a bitufted morphology with a very elaborate dendritic tree. Martinotti cells always express SOM and never express PV or VIP (Defelipe, 2002, Markram et al., 2004, Druga, 2009).

Axon targeting interneurons

Chandelier cells are the only axon targeting interneurons, allowing them to override all prior signal integration to modify the action potential output of the pyramidal cell (Freund, 2003, Taniguchi et al., 2013). They possess smooth dendrites and a unique characteristic axon with terminal boutons that resemble candlesticks (DeFelipe, 1999). The terminal boutons innervate only the initial segments of pyramidal cell axons and never innervate other interneurons. Chandelier cells are found in layers II–VI of the neocortex, and typically express one or both of the calcium-binding proteins PV and CB, but not CR (Inan and Anderson, 2014).

1.2.2.2.1. *Funtional classification of interneurons*

Interneurons possess a wide range of active and passive membrane properties and this has allowed researchers to use their response to depolarization as a way to characterize or define specific subpopulations. According to their intrinsic firing characteristics interneurons can be classiffied as fast spiking (FS) cells, burst-spiking non-pyramidal (BSNP) cells, regular-spiking non-pyramidal (RSNP) cells, late-spiking (LS) cells and irregular-spiking (IS) cells (Fig 1.4).

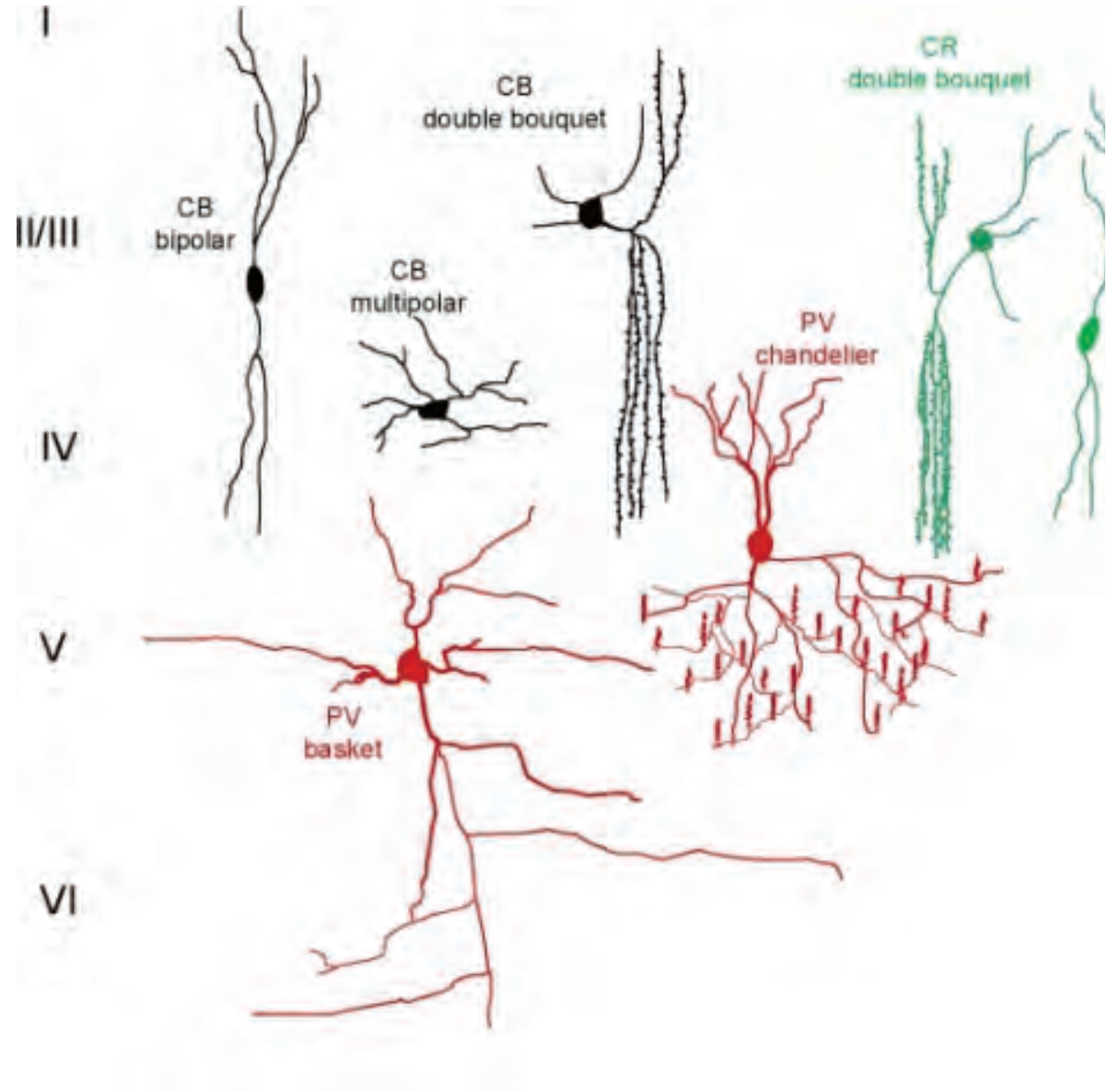


Figure 1.3. Inhibitory interneurons in the neocortex.

Schematic representation of the major interneuron subtypes that populate different layers of the cortex (Adapted from Raghanti et al 2010).

Fast spiking interneurons

FS interneurons in the cortex are typically PV-expressing basket and chandelier cells (Kawaguchi et al., 1987). When depolarized to threshold these cells generate a train of action potentials at high frequency, each with a large and fast after hyperpolarization. These neurons show little spike frequency adaptation, have a lower input resistance than all other subtypes of interneurons, and are generally more hyperpolarized at rest (Kawaguchi and Kubota, 1996). Due to their prevalence throughout the cortex and the magnitude of their response, FS cells are thought to be key regulators of the excitatory-inhibitory balance in the cortex.

Burst spiking non-pyramidal and regular spiking non-pyramidal interneurons

The burst spiking non-pyramidal and the regular spiking non-pyramidal firing patterns are associated with the Martinotti, double bouquet and bipolar cells. The burst spiking non-pyramidal interneurons show a typical burst-like discharge with two or more low threshold spikes from hyperpolarized potentials. They represent mainly SOM expressing Martinotti cells of layer V that can co-express CB and also VIP expressing double bouquet cells in layer II/III and V. SOM- and VIP-expressing burst spiking non-pyramidal neurons produce longer duration action potentials, have a higher input resistance and a more depolarized resting membrane potential than fast-spiking interneurons (Kawaguchi and Kubota, 1996, 1997).

The regular spiking non-pyramidal cells discharge in a similar manner to regular spiking pyramidal cells. Regular spiking non-pyramidal neurons have been recorded in layers II/III and V, and have been identified as SOM-expressing Martinotti and VIP-expressing double bouquet and bipolar cells in layers II/III and V (Kawaguchi and Kubota, 1996, 1997, 1998). Usually in SOM-expressing regular spiking non-pyramidal cells depolarization only induces a depolarizing hump with a single spike (Kawaguchi and Kubota, 1996).

Late spiking (LS) cells

Late-spiking (LS) cells discharge with a considerable delay after a depolarizing step near threshold. These cells are found in layers II/III and V, and have been identified as neurogliaform cells (Kawaguchi et al., 1987, Gupta et al., 2000).

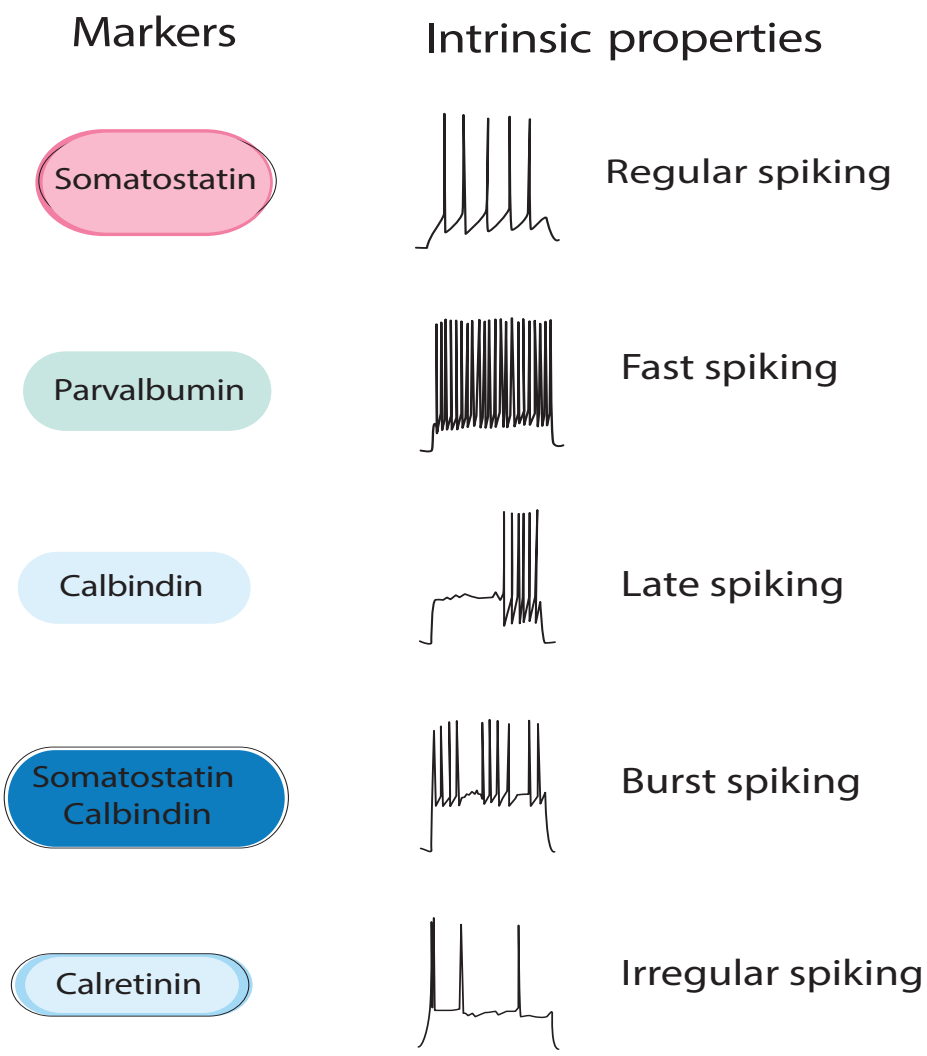


Figure 1.4. Firing characteristics of inhibitory interneurons in the neocortex.

Interneuron cell types defined by a combination of marker expression and intrinsic firing properties (Adapted from Kepecs and Fishell, 2014)

Irregular spiking (IS) cells

Irregular spiking (IS) cells are the small fraction of vertically oriented bipolar interneurons within layers II/III and V that express CR or VIP (Cauli et al., 1997). They fire an initial burst of action potentials, between two and six depending on the level of depolarization, followed by irregularly spaced action potentials. They have the highest input resistance of all interneuron subtypes (Kawaguchi and Kubota, 1996, Cauli et al., 1997, Vucurovic et al., 2010).

The diversity of inhibitory interneurons and synapses, as well as a balance between excitatory and inhibitory connections, are crucial for normal neocortical function. The complexity and specificity of neuronal connections renders the brain particularly vulnerable to external insults such as TBI. Post-injury alterations in the excitatory and complex inhibitory circuits have not been fully elucidated and need further investigation.

1.3. THE NEURONAL RESPONSE TO INJURY

1.3.1. Axonal injury

Axonal injury is a common consequence of TBI. Pyramidal cell axons are particularly vulnerable to injury as they project over long-distances and have a complex and highly organized structure (Meythaler et al., 2001, Johnson et al., 2012). Axonal injury can be a consequence of a primary injury or can occur as a secondary effect. Primary axonal injury or primary axotomy is the result of a rapid and direct disconnection of the axon into two separate segments at the moment of injury (Blennow et al., 2012). Axonal degeneration can also be a consequence of a series of events that take place after a primary injury known as secondary axonal injury or diffuse axonal injury (DAI). DAI is considered a progressive event, gradually evolving from a focal axonal alteration to a delayed axonal disconnection (Buki and Povlishock, 2006). Animal and human studies indicate that secondary axotomy is the most common form of axotomy after TBI (Povlishock, 1992, Christman et al., 1994).

DAI is initiated by the disruption of axonal transport that leads to axonal swelling and ends with disconnection or secondary axotomy and Wallerian degeneration of the distal segment of the axon (Maxwell et al., 1990). The gold standard for the pathological identification of DAI is the detection of Amyloid Precursor Protein (APP). APP has been observed to accumulate rapidly after an

injury forming typical spheroid-shaped APP^{+} axonal swellings (Gentleman et al., 1993, Sherriff et al., 1994, Buki and Povlishock, 2006). A recent study indicates that a range of different phenotypes of axonal pathologies can be found after axonal injury with variable labeling for APP, and some of these morphologies not only represent different stages of an evolving pathology, but are related to specific axonal compartments and even to different neuronal subtypes (Hanell et al., 2015a). Therefore the axonal response to injury requires further investigation as understanding this process may enable the development of novel treatments to target primary and secondary axon degeneration.

1.3.1.1. Injury related alterations to the cytoskeleton

The cytoskeleton is the underlying scaffold maintaining neuronal structure. It consists of an array of fibrous proteins that are dynamic and enable diverse cellular processes such as cell division, intracellular transport and cell migration (Fletcher and Mullins, 2010). The axonal cytoskeleton is highly vulnerable to injury, and it is now recognized that even in the absence of initial axonal transection, and in seemingly mild forms of TBI, adverse alterations occur at the level of the axonal cytoskeleton that have disastrous consequences for neuronal function (Smith et al., 2013). Cytoskeletal alterations such as neurofilament compaction and disruption, and the disassembly of microtubules, are thought to lead to defective axonal transport and swelling that are characteristic features of DAI (Smith and Meaney, 2000). After TBI there can be a disruption to the cell membrane surrounding the axon, which is called the axolema, and this disruption leads to Ca^{++} influx. Even though the primary mechanism leading to extracellular calcium influx is largely understood for large caliber axons, in fine caliber, non-myelinated neurons the source of local calcium is not related to axolema disruption, but rather to the activation of Na^{+} channels that causes depolarization. This results in Ca^{++} influx through the activation of voltage-gated Ca^{++} channels and reversal of the Na^{+}/Ca^{++} exchange pumps (Wolf et al., 2001). Some data also implicate voltage-gated L- and T-type calcium channels and the release of Ca^{++} from intracellular stores in facilitating the post-injury influx of Ca^{++} (Knoferle et al., 2010, Stirling et al., 2014). Regardless of the mechanism, Ca^{++} influx causes axonal damage, destruction of the axonal cytoskeleton, mitochondrial dysfunction and ultimately cell death. The intensive sequestration of extracellular Ca^{++} opens the mitochondrial membrane permeability transition (MPT)-pore (Zoratti and Szabo, 1995, Kim et al., 2003, Mazzeo et al., 2009). Induction of the MPT-pore results in mitochondrial swelling, axon disconnection and finally cell death through

the MPT release of pro-apoptotic enzymes such as cytochrome-c and apoptosis inducing factor (Cai et al., 1998, Bernardi and Forte, 2007, Lemasters et al., 2009) (Halestrap, 2009). Furthermore, mitochondrial dysfunction is related to energy-failure and results in dysfunction or failure of ionic pumps that leads to uncontrolled ionic homeostasis, further mitochondrial swelling and neuronal death (Liu et al., 2009).

The axolemal disruption has also been linked to the activation of cysteine proteases and the proteolytic degradation of the cytoskeletal network (Buki and Povlishock, 2006, Johnson et al., 2012). Two important members of the cysteine protease family are the calpains and caspases. Caspases are a well-described group of cysteine proteases with a characteristic function in apoptosis. Calpains, on the other hand, are considered to play an important role in necrosis and apoptosis (Harwood et al., 2005). The activation of these two groups of proteases worsens outcomes after injury as they actively participate in the degradation of the local axonal cytoskeleton resulting in local axonal failure and disconnection (Ma, 2013).

Microtubule disruption following injury can have severe effects on neuronal structure and function. The loss and misalignment of microtubules plays an important role in degeneration, while preventing regeneration of the injured neuron. In particular it is recognized that the post-injury calcium influx affects microtubule disassembly and leads to a loss of microtubule associated proteins (MAPs) such as tau and MAP-2 (Smith et al., 1999, Smith and Meaney, 2000, Farkas and Povlishock, 2007, Tang-Schomer et al., 2010).

The importance of microtubule deregulation in TBI pathology suggests that treatment with compounds that stabilize cytoskeletal microtubules after injury can potentially modify multiple aspects of the brain's response to trauma and improve outcomes after brain injury (Brunden et al., 2010, Hellal et al., 2011, Sengottuvel et al., 2011, Baas and Ahmad, 2013b, Hur et al., 2014). Recent publications have presented proof-of-principle experiments indicating that the microtubule-stabilizing drug Paclitaxel, widely used for cancer treatment, can enhance axon regeneration, improve function and reduce glial scarring after injury (Hellal et al., 2011, Sengottuvel et al., 2011). However, a more recent study has found no axon regeneration or functional recovery after spinal contusion injury with the use of the same drug (Popovich et al., 2014). The discrepancy in these observations reinforces the fact that a more comprehensive and mechanistic understanding of the neuronal response to injury, and more specifically microtubule dynamics after injury, is needed before microtubule-stabilizing drugs can move forward as a realistic post-injury drug

treatment.

Neurofilaments provide tensile strength and establish neuronal structure and are also frequently altered by TBI (Hall and Lee, 1995, Posmantur et al., 2000, Smith and Meaney, 2000, Siedler et al., 2014). Neurofilament disruption occurs within minutes of the injury and can persist for hours, causing impaired axonal transport and swelling. Three principal proteins compose the neurofilaments, NF light (NF-L), medium (NF-M), and heavy (NF-H) proteins, with NF-M and NF-H including side arms of different lengths. These side arms may be phosphorylated and the extent of this phosphorylation is related to the diameter of axons (Hisanaga and Hirokawa, 1990, Hall and Lee, 1995). Neurofilament compaction is often attributed to proteolysis of the neurofilament side arms (Povlishock et al., 1997, Okonkwo et al., 1998). It is hypothesized that this could be the consequence of an imbalance between protein kinases and phosphatases, or could result from disrupted calcium homeostasis and the abnormal activation of proteolytic calpains that cleave the neurofilament side arms (Buki et al., 2003, Ma, 2013). The overall consequence is the reduction of intra-filament spacing and neurofilament compaction (Christman et al., 1997, Smith et al., 2003, Johnson et al., 2013).

Another characteristic of neurofilament dysfunction after TBI is their aberrant phosphorylation. Neurofilaments are normally maintained in a dephosphorylated form in the neuronal cell body and dendrites and are phosphorylated in the axons (Maxwell and Graham, 1997). Neurofilament phosporylation regulates axonal transport, such that hypophosphorylated neurofilaments are transported more quickly than phosphorylated neurofilaments (Shea et al., 2003). After TBI, phosphorylated neurofilaments accumulate in the cell body of neurons, which impedes the transport of neurofilaments to the axons and decreases axon caliber after TBI (Huh et al., 2002, Hamberger et al., 2003).

1.3.2. Interneurons and their response to injury

Several studies have demonstrated that the adult CNS retains a significant capacity for structural plasticity in response to alterations in sensory experience or after injury. Neuronal plasticity is defined in terms of alterations in synaptic strength and wiring, axonal sprouting, remodeling of dendritic arbors and changes in dendritic spine and axonal bouton turnover (Florence, 1998, Grutzendler et al., 2002, Hickmott

and Steen, 2005, Holtmaat et al., 2005, Lee et al., 2006, Lee et al., 2008, Campbell et al., 2012). Looking specifically at the interneurons and their capacity for dendritic arbor remodeling, Lee et al. (2006, 2008) indicated that inhibitory neurons are capable of significant dendritic remodeling under normal physiological conditions. They demonstrated that in the visual cortex GABA-positive non-pyramidal interneurons exhibit dynamic arbor rearrangements while pyramidal cells remain stable. These results indicate that dendritic structural remodeling is an example of adult plasticity and that circuit rearrangements in the adult cortex are restricted to specific cell types (Lee et al., 2006). Further studies proved that the interneurons that remodel their dendrites are contained within a dynamic zone corresponding to a superficial band of layers II/III. Furthermore, dendritic remodeling is not inherent to a specific interneuron subclass, suggesting that the capacity of interneurons to rearrange their neurites is not determined by genetic lineage, but rather imposed by cortical laminar circuitry (Lee et al., 2008).

The dendritic remodeling of interneurons has also been studied under pathological conditions. Blizzard et al. (2011) demonstrated that a population of interneurons respond to focal structural injury in a manner that is entirely different to principal projection neurons. Following a discrete unilateral lesion to the adult rodent somatosensory cortex, CR positive interneurons remodeled their dendrites away from the injury site, while the regenerative response of pyramidal neurons was the opposite, sprouting axons into the site of injury (Blizzard et al., 2011). How other subpopulations of interneurons respond to injury, and how this response differs with injury type is currently under investigation. Further research is required to gain a better understanding of the role of interneurons in modulating degeneration and regeneration of the nervous system after TBI. Furthermore, the unique response of interneurons to injury could make them novel targets for therapeutic interventions aimed at minimizing secondary pathologies.

1.4. NEURONAL REGENERATION AND REPAIR FOLLOWING TBI

Complete functional recovery after an injury to the adult brain would be predicted to require the replacement or repair of dead or damaged cells in the injury site and/or the compensatory remodeling of surrounding intact cells to bypass the damaged circuitry. The two main mechanisms by which lost neuronal connections and degenerated neurons can be replaced are axonal regeneration and neurogenesis. Contrary to the common belief that mature neurons are incapable of

significant regeneration, a number of studies have demonstrated that damaged axons initially react to the injury and undergo regenerative sprouting (King et al., 2001, Dickson et al., 2007). This indicates that mature neurons have an intrinsic capacity to adaptively respond to damage and to attempt regeneration (King et al., 2000, Chuckowree and Vickers, 2003, Blizzard et al., 2009, Blizzard et al., 2011). It remains to be shown, however, that regeneration can be achieved by promoting spouting response at or around the injury site or that this response is beneficial and promotes functional recovery. There is some evidence indicating that post-injury sprouting may not always be functionally appropriate, with potentially aberrant axonal connectivity contributing to the development of epilepsy following brain trauma (Salin et al., 1995, McKinney et al., 1997).

In terms of neuron replacement, the adult brain has two major stem cell niches, the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone of the dentate gyrus of the hippocampus. The SVZ in particular is a known site of neurogenesis where neural progenitor cells are present and are capable of differentiating into neurons, astrocytes and oligodendrocytes (Romanko et al., 2004). There is evidence suggesting that after an injury to the brain the rate of cell proliferation is altered. Some studies indicate that this injury-induced cell proliferation could result in the generation of new neurons and/or glial cells (Gu et al., 2000, Magavi et al., 2000, Arlotta et al., 2003, Chen et al., 2003). After TBI the potential manipulation of these progenitor cell populations could result in newly produced cells that could compensate for the significant neuronal cell loss.

1.4.1. Axonal regeneration versus axonal sprouting

Axonal regeneration refers to successful restoration of the contact between the axotomised neuron and its original neuronal target. For complete regeneration to occur there should also be remyelination of the damaged axon, and the reestablishment of functional synapses (Horner and Gage, 2000, Tuszynski and Steward, 2012). Damaged axons in the adult mammalian peripheral nervous system (PNS) and the developing CNS are able to fully regenerate after injury (Ferretti et al., 2003). The regenerative ability of adult PNS neurons is achieved by the reactivation of an embryonic growth program that involves changes in gene expression related to a rapid axonal growth that leads to functional regeneration (Smith and Skene, 1997, Makwana and Raivich, 2005).

On the other hand damaged neurons from the adult CNS do not fully

regenerate but usually 'sprout' after injury. Sprouting describes a process related to any outgrowth from cut or intact fibers that spans moderate distances and leads to the formation of multiple local axon collaterals that do not usually result in the restoration of normal connectivity (Horner and Gage, 2000, Cafferty et al., 2008, Tuszynski and Steward, 2012). The inability of the adult mammalian CNS to regenerate after injury is related to intrinsic cell-autonomous factors as well as extrinsic factors within the environment of the mature brain, such as the presence of myelin-associated inhibitors and the formation of the glial scar (Silver and Miller, 2004, Yiu and He, 2006, Huebner and Strittmatter, 2009).

1.4.2. Extrinsic barriers to axon regeneration

1.4.2.1. Myelinating cells and myelin-associated inhibitors

Myelin plays a key role in limiting post-injury axonal outgrowth in the CNS. It has been well described that after an injury neurons are exposed to myelin debris, which is inhibitory to axonal growth. It is believed that the post-injury regenerative abilities of PNS and CNS neurons is at least partially due to the PNS and CNS being myelinated by different cell types. There is evidence that CNS myelin, unlike PNS myelin, can be a source of axonal outgrowth inhibition since CNS myelin expresses myelin-associated inhibitory factors (Schwab and Thoenen, 1985, Yiu and He, 2006). Furthermore, in the PNS there is limited exposure of severed axons to the inhibitory myelin environment, as Schwann cells and macrophages rapidly clear myelin debris from the injury site (Reichert et al., 1994, Martini et al., 2008). In the CNS there is a slower activation and recruitment of macrophages after injury, which is reliant on the blood brain barrier (BBB) being compromised, and this could account for the differences in the post-injury environment that leads to limited axonal regeneration in the CNS (Lotan and Schwartz, 1994, Hirschberg and Schwartz, 1995, Rapalino et al., 1998). However, this is controversial as other studies indicate that the number of activated macrophages after injury can be similar in the PNS and CNS, and that differences in regeneration are mainly related to the myelinating cells themselves (Leskovar et al., 2000).

In this regard, it has been demonstrated that there are intrinsic differences between Schwann cells and oligodendrocytes, with respect to their response to injury (Filbin, 2003, He and Koprivica, 2004, Yiu and He, 2006). These cells play a key role after neuronal injury in creating a growth-promoting environment in the PNS and a growth inhibiting environment in the CNS (Allen and Barres, 2009). In the PNS after

injury, Schwann cells de-differentiate and down regulate all myelin proteins so that they become permissive to axonal regeneration (Filbin, 2003). On the other hand, in the CNS, oligodendrocytes do not down regulate myelin proteins but instead continue to express them after injury thereby limiting axonal growth (Sivron and Schwartz, 1994). Additionally, there is very little death of Schwan cells after axonal injury and this allows for an active role of these cells in axonal regeneration forming new myelin conduits to guide and nurture regenerating axons (Brosius Lutz and Barres, 2014). In the CNS there is widespread loss of oligodendrocytes after injury. Moreover, there is evidence suggesting that the remaining uninjured oligodendrocytes have very little capacity to promote axonal regeneration and remyelination (Keirstead and Blakemore, 1999). Furthermore, oligodendrocytes have been linked to growth inhibition following injury due to the release of growth inhibitory factors or myelin associated inhibitors.

There are a number of myelin associated axonal growth inhibitors described in the literature but among them the myelin proteins Nogo, myelin associated glycoprotein (MAG) and the oligodendrocyte myelin glycoprotein (OMgP) have been studied in more detail since they represent the main proteins associated with growth inhibition (Brosius Lutz and Barres, 2014). The Nogo inhibitory factor has three different isoforms (Nogo-A, -B, and -C). Nogo-A is mainly expressed in the nervous system, while Nogo-B and -C are widely expressed outside the CNS (GrandPre et al., 2000). Nogo-A is enriched in oligodendrocytes in the endoplasmic reticulum and on the cell surface of the myelin sheath (Fujita and Yamashita, 2014). It has a pathologic role in limiting axon regeneration post-injury with two domains that are inhibitory to axonal growth, Nogo 66 and amino-nogo (Chen et al., 2000, Huber and Schwab, 2000, Prinjha et al., 2000, GrandPre et al., 2002). The MAG is a transmembrane protein associated with myelin formation and maintenance. MAG can promote axonal growth during the neonatal period but becomes inhibitory after birth. After axonal injury myelin damage leads to the release of a soluble proteolytic form of the protein that inhibits axonal regeneration (Tang et al., 2001, Wong et al., 2002, Yiu and He, 2006). The oligodendrocyte myelin glycoprotein is a glycosyl phosphatidyl-inositol (GPI)-linked protein. It is a relative minor component of myelin that is expressed not only by oligodendrocytes but also by neurons. All these myelin associated inhibitory factors have been shown to bind to the same Nogo receptor on axons and form a complex with the P75 nerve growth factor receptor (NgR-P75) (Fournier et al., 2001, Wang et al., 2002a). This complex signals via the small intracellular GTPase, RhoA. RhoA activates ROCK and triggers downstream

effectors that regulate cytoskeletal reorganization, growth cone collapse and neurite outgrowth inhibition (Lehmann et al., 1999, Fujita and Yamashita, 2014).

1.4.2.2. Glial scar

Mature astrocytes respond to injury through a complex series of events globally referred to as reactive gliosis (Sofroniew, 2005). Reactive astrocytes form a glial scar at the lesion site that helps isolate the injury and minimize the area of inflammation and cellular degeneration, although it becomes a barrier to axon regrowth (Silver and Miller, 2004, Yiu and He, 2006). The glial scar is not only a physical barrier for axonal regeneration but also contains molecules that impede axon outgrowth. Chondroitin sulfate proteoglycans (CSPGs) are upregulated by reactive astrocytes after CNS damage and are both membrane bound and secreted into the extracellular space, forming a concentration gradient along the injury (Huebner and Strittmatter, 2009). There is a very high concentration of inhibitory molecules at the center of the injury site and regenerating axons eventually become dystrophic and fail to regenerate near the lesion epicenter as a result of an inhibitory and non-growth conducive environment (Toy and Namgung, 2013).

Many studies in the past have focused largely on characterizing the growth inhibitory molecules in the CNS, (Schwab, 1990, Silver and Miller, 2004, Case and Tessier-Lavigne, 2005, Yiu and He, 2006, Yamashita and Fujita, 2014) and significant effort has been made to block these inhibitory molecules by genetic and pharmacological approaches in order to promote axonal growth (David and Aguayo, 1981, Schnell and Schwab, 1990, Schwab, 1992, Bregman et al., 1995, Bandtlow and Schwab, 2000, GrandPre et al., 2002, Tauchi et al., 2012, Carballo-Molina and Velasco, 2015). However, blocking these inhibitory molecules only lead to limited regeneration, and the majority of the adult neurons still failed to regenerate their axons (Schwab, 1992, Kim et al., 2004, Zheng et al., 2005). This supports the idea that extrinsic inhibitory factors as well as intrinsic cell-autonomous mechanisms are involved in the regenerative failure of injured adult CNS neurons.

1.4.3. Intrinsic or cell-autonomous axon regeneration mechanisms

In vivo and *in vitro* data support the concept that neuronal maturation is associated with the gradual loss of the intrinsic growth capacity of the neuron (Brundin et al., 1988, Wigley and Berry, 1988, Fawcett et al., 1989, Hankin and Lund, 1990, Shimizu et al., 1990, Liu et al., 2011). The growth-associated protein 43 (GAP-43), the phosphatase and tensin homolog (PTEN) and the cyclic adenosine

monophosphate (cAMP) are among the factors that have emerged as regulators of the growth state of embryonic and PNS neurons and have been involved in the lack of regeneration of the adult CNS after injury (Tuszynski and Steward, 2012). GAP-43 regulates cytoskeletal remodeling in the neuronal growth cone, a process fundamental for axon elongation. Its expression is high during development, declines through adulthood and increases during regeneration (Jacobson et al., 1986, Stein et al., 1988). In the PNS there is a steady increase in the expression of GAP-43 that is related to a phase of axonal elongation and regeneration (Yang and Yang, 2012). By contrast, in the adult CNS, a weak and transient expression of the protein relates to a spontaneous sprouting response that does not usually lead to functional recovery (Aigner et al., 1995).

Another intrinsic factor involved in the regulation of neuronal growth and post-injury regeneration is PTEN. PTEN is a negative regulator of axon growth that shows increased expression in adult neurons and suppresses the activity of the mammalian target of rapamycin (mTOR) (Krishnan and Zochodne, 2014). mTOR is a protein kinase that regulates protein translation, cell growth and size (Hay and Sonenberg, 2004). It has been demonstrated that the deletion of PTEN activity can increase the capacity of adult neurons to regenerate their axons after injury (Park et al., 2010, Lee et al., 2014). Previous studies have shown that the genetic depletion of PTEN activity promotes robust axon regeneration after optic nerve injury in adult retinal ganglion cells (RGCs) (Park et al., 2008). Furthermore, studies in injured adult corticospinal neurons indicate that PTEN deletion enables the mounting of a robust regenerative response after spinal cord injury (Liu et al., 2010). PTEN signaling is also involved in oligodendrocyte-axon interactions and is important for myelin integrity. Even though the inhibition or deletion of PTEN has been shown to have beneficial effects on neuronal regeneration, the chronic activation of the mTOR pathway in oligodendrocytes can have negative consequences for axonal integrity and myelination (Harrington et al., 2010).

The cyclic adenosine monophosphate (cAMP) has also been shown to be a growth-promoting factor inducing axonal growth and regeneration. In the PNS after neuronal injury there is activation of the membrane-bound adenylyl cyclase that converts ATP into the second messenger cyclic AMP (Makwana and Raivich, 2005, Navarro et al., 2007). The production of cyclic AMP alters the physiology of the neuron, changing its function from one of neurotransmission to one of growth and regeneration, activating a series of down-stream effectors (Yaniv et al., 2012, Knott

et al., 2014). In the CNS elevated endogenous cAMP, as well as a decrease in its degradation, have been shown to induce axonal regeneration after injury (Bhatt et al., 2004, Pearse et al., 2004, Lau et al., 2013).

Overall it is likely that a complete functional recovery following a traumatic injury to the adult brain would require the replacement of lost and dying neurons, the regeneration of lost axons, and their reconnection to synaptic targets. The plastic response of neurons to injury, that determines the brain's potential for compensation and recovery, has not been fully elucidated. Understanding how specific subpopulations of neurons respond to injury is crucial in order to try to manipulate the injured brain's potential for plasticity and facilitate desirable outcomes after injury.

1.5. THESIS AIMS

One important factor underlying the lack of effective strategies to treat TBI is an incomplete understanding of the fundamental cellular processes activated by an injury to the brain. Relatively few studies have directly addressed the role of interneurons in the degenerative and regenerative responses evoked by TBI. The primary objective of this thesis is to further investigate the unique response of subpopulations of neurons to injury in order to gain a better understanding of the events that take place after damage to the adult brain and potentially help develop effective therapeutic strategies. The current thesis is based upon the hypotheses that recovery following trauma will require the activation of reactive and compensatory plasticity and that the mechanisms underlying this plasticity are intrinsically different for excitatory and inhibitory cortical networks.

Aim 1: To determine if different neuronal subpopulations are selectively vulnerable in a model of *in vitro* axonal injury.

A full understanding of the neuronal response to injury is vital for devising successful post-injury treatments. To date, a comprehensive study focusing specifically on neuronal subtype specific responses to injury has not been performed. Chapter 2 of this thesis aimed to investigate excitatory and inhibitory neuron vulnerability to injury using an *in vitro* model of transection injury, in combination with immunocytochemical and electrophysiological techniques.

Aim 2: To investigate interneuron responses to injury in a clinically relevant *in vivo* model of focal and diffuse injury.

To date, the literature regarding the susceptibility of interneurons to injury remains inconclusive. Given the importance of appropriate interneuron function for providing balanced inhibition in the cortex, it is imperative that the extent of interneuron damage is evaluated following traumatic injury. In Chapter 3 of this thesis a rodent model was utilized to determine the effects of traumatic injury on specific subpopulations of interneurons and the induction of post-injury interneuron regeneration. Furthermore, post-injury functional studies were performed to investigate changes in inhibitory synaptic input.

Aim 3: To use an *in vitro* platform of injury to test microtubule stabilization as a pharmacological intervention for the treatment of TBI.

Microtubule stabilizing drugs, such as epothilones, are now being investigated for their potential use in treating diseases of the nervous system. The importance of microtubule dysregulation in TBI pathology suggests that post-injury, treatment with compounds that stabilize cytoskeletal microtubules may modify multiple aspects of the brain's response to trauma and improve outcomes after TBI. In Chapter 4 the effect of Epothilone D (Epo D) on the post-injury axonal sprouting of mature cultured cortical neurons was examined. The response of regenerating axons to Epo D treatment was analyzed using immunocytochemistry, live imaging and molecular techniques.

2. AXONAL TRANSECTION IN MATURE CORTICAL NEURONS INDUCES CELL SPECIFIC DENDRITIC REMODELING

2.1 INTRODUCTION

Accumulating evidence indicates that the brain can undergo substantial reorganization after injury. The capacity of the brain to adapt after structural injury is globally referred to as post-injury plasticity. Plastic changes after injury can include alterations in synaptic strength, remodeling of axonal and dendritic arbors and neurogenesis (Keyvani and Schallert, 2002, Chuckowree et al., 2004, Carmichael, 2006, Dancause and Nudo, 2011, Hunt et al., 2011, Canty et al., 2013, Perederiy and Westbrook, 2013). The degree of plasticity occurring after injury has been classically linked to age, mode of injury, and/or the extent and region of injury (Horner and Gage, 2000, Chen et al., 2002, Cafferty et al., 2008). More recent studies suggest that post-injury plasticity can also be dependent on the subpopulation of neurons affected, specifically in terms of dendritic remodeling (Blizzard et al., 2011, Canty et al., 2013), consequently implying that a particular response to injury can be intrinsic to a specific neuronal subtype. These results indicate that a broader understanding of the response of different subpopulations of neurons to structural injury and the possible mechanisms underlying these responses is needed.

Dendrites are the structures responsible for receiving and processing neuronal inputs. Their morphology, in terms of branching pattern, determines the number and type of inputs a neuron can receive (Macias, 2008). During early postnatal development neuronal dendritic branching patterns can be significantly remodeled and shaped by experience and learning during critical periods (Bavelier et al., 2010). There is extensive evidence indicating that inhibitory interneurons play a crucial role in neuronal plasticity as an increase in cortical inhibition regulates the onset and closure of critical periods (Hensch, 2005, Di Cristo et al., 2007, Baroncelli et al., 2011, Chen et al., 2011). However, in adulthood most dendritic arbors remain virtually stable in the naïve brain. This has been described in a number of relevant imaging studies where no dendritic arbor structural changes were found in pyramidal neurons in any region of the cortex (Trachtenberg et al., 2002, Mizrahi and Katz, 2003). In contrast to excitatory pyramidal neurons, non-pyramidal inhibitory interneurons seem to be more plastic and exhibit a range of dendritic structural modifications when imaged over time in the primary visual cortex (Lee et al., 2006, Lee et al., 2008) and other regions of the cortex (Chen et al., 2011). While the role of

inhibition during development is increasingly well understood, recent studies have indicated that changes in the inhibitory circuitry are also involved in regulating dendritic plasticity in adulthood. Furthermore, it has been suggested that morphological modifications in inhibitory cell axons and dendrites after sensory deprivation can precede and drive the changes observed in the excitatory circuitry (Yazaki-Sugiyama et al., 2009, Marik et al., 2010, Keck et al., 2011, Chen and Nedivi, 2013).

Given the important part that interneurons play during developmental plastic periods and their involvement in plasticity after sensory deprivation, the aim of this Chapter was to gain insights into the possible role of inhibitory neurons in plastic changes after traumatic injury. To facilitate the investigation of the differential response of excitatory and inhibitory neurons to structural injury an *in vitro* model of injury was developed. Post-injury plasticity was explored in terms of morphological changes in the dendritic arbor, focusing specifically on a subpopulation of Calretenin expressing interneurons. The Calretinin population of interneurons was selected for this study as they have the capacity to remodel their processes after injury (Blizzard et al., 2011). In the current investigation, modifications in their dendritic arbor were investigated in detail at different time points post-injury, at increasing distances from the injury site, and were compared with changes in other populations of interneurons and also pyramidal cells.

The *in vitro* model of axonal injury was used to investigate the intrinsic response of excitatory and inhibitory neurons to structural injury. Primary cortical neuronal cultures have been extensively used for the study of neurotransmission, neurodegeneration, and neurodevelopment (Morrison et al., 2011, Gordon et al., 2013). Despite being widely utilized, a comprehensive analysis of the functional development of different populations of neurons and the electrophysiological characteristics of excitatory and inhibitory neurons in culture had not been undertaken. In the current study, the electrophysiological characterization of neurons *in vitro* allowed the evaluation of not only morphological subpopulation-specific post-injury alterations, but also important insight into functional changes.

2.2. MATERIALS AND METHODS

2.2.1. Wild type and transgenic mice

All experimental procedures involving animals were approved by the Animal Ethics Committee for animal experimentation of the University of Tasmania (A0011952) and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. *Thy1-YFP* transgenic mice (B6; CBA-Tg *Thy1-YFP* GJrs / GfngJ) were obtained from the Jackson Laboratory (Bar Harbor, ME). *GAD67-GFP* mice were developed by Prof. Nobuaki Tamamaki (Kyoto University) and were a kind donation from Prof. John Bekkers (Australian National University). *GAD67-GFP mice* are on a C57BL6/J background and express green fluorescent protein (GFP) in neurons expressing GAD67, one of the two genes that encode isoforms of the GABA-synthesizing enzyme, glutamic acid decarboxylase (Tamamaki et al., 2003, Suzuki and Bekkers, 2010). Animals were bred at the University of Tasmania. Animals were housed in individually ventilated cages, maintained at ~20^{o}C, on a 12 hour light/dark cycle, with access to food and water *ad libitum*.

2.2.2. Cell culture

Primary dissociated cortical neuron cultures were prepared from mouse embryos as previously described (Dickson et al., 2000). Briefly, *Thy1-YFP* and *GAD67-GFP* transgenic mice were time-mated, the pregnant females killed by CO_2 exposure at 15.5 days of gestation, and the embryos removed. Embryos were genotyped by fluorescence imaging using 470nm light on a Carestream Image station 4000MM pro (Carestream Molecular Imaging, USA). Neocortical hemispheres were dissected into 5ml Hanks Buffered Salt Solution (HBSS; Thermo-fisher Scientific, USA). Cortical tissue was dissociated by enzymatic digestion (0.025% w/v Trypsin, 5 minutes, 37^{0}C), which was halted by the addition of 1ml of pre-warmed medium [Neurobasal^{TM} supplemented with 2% v/v B27, 10% v/v fetal calf serum, 0.5mM L-glutamine, 25µM glutamate and 1% streptomycin penicillin; Thermo-fisher Scientific, USA]. Tissue pieces were allowed to settle and after removal of the medium, dissociation was completed by resuspension of the cells in 500µl of pre-warmed medium and gentle trituration. Cell viability and concentration was assessed using trypan blue vital dye exclusion. 3.5 x 10^4 cells/mm^2 were plated per 19mm diameter glass coverslip (Marienfeld, Germany). Each coverslip was pre-coated with 0.001% Poly-L-lysine (Sigma-Aldrich, USA) in 0.01M PBS (pH 7.0).

Cultures were maintained in a 37°C humidified atmosphere of 5% CO_2 for up to 15 days. At 1 day *in vitro* (DIV) the medium was removed and replaced with serum-free growth medium [Neurobasal™, 2% v/v B-27 supplement, 0.5mM L-glutamine, and 1% streptomycin penicillin; Thermo-fisher Scientific, USA], after which time ~50% of the medium was replaced every 3–4 days.

2.2.3. Electrophysiology

Cortical neurons cultured on glass coverslips for 7 DIV or 15 DIV, were superfused with bicarbonate-buffered solution [155mM NaCl, 3.5mM KCl, 1mM NaH_2PO_4, 26.2mM $NaHCO_3$, 1.4mM $MgCl_2$, 2.4mM $CaCl_2$, and 11mM glucose; osmolarity adjusted to 295-305mOsm/kg by the addition of D-sorbitol (all Sigma)]. A single cell expressing GFP or YFP was whole-cell voltage-clamped and was filled with Alexa Fluor-568 dye. Electrophysiological data for analysis were obtained from neurons (at room temperature) that had a resting membrane potential (RMP) below -40mV. Voltage-gated sodium and potassium currents were elicited with a series of voltage steps (from -70 to 50mV, in 10mV increments) from a holding potential of -70mV. The peak net inward current (reflecting the difference between the inward I_{Na} and the other voltage-dependent outward currents) was quantified after subtracting the capacity current and an assumed ohmic leak current, scaled from the response to hyperpolarizing pulses (as per (Clarke et al., 2012). Voltage responses to current injection were recorded from the cell's resting potential (applying 25pA steps for 200ms from -100 to 500pA). After recording, the patch electrode was removed from the cell, where possible leaving the soma intact for imaging.

Patch electrodes formed from borosilicate glass capillaries (1.5mm O.D. x 0.86mm I.D, Harvard Apparatus, UK) with a resistance of 5-7MΩ were filled with a solution containing: 130mM K-gluconate, 4mM NaCl, 10mM HEPES, 0.5mM $CaCl_2$, 10mM BAPTA, MgATP 4mM, Na_2GTP 0.5mM, pH set to 7.4 with KOH, and osmolarity adjusted to 290mOsm/kg with D-sorbitol. An Axopatch 200B amplifier (Molecular Devices, USA) was used for voltage- and current-clamp. Data were adjusted to account for the electrode-junction potential. Data were sampled at 10 kHz and filtered at 5 kHz using pClamp 9.2 software, and offline analysis was performed using Igor (WaveMetrics) and Axograph scientific software.

2.2.4. Axonal transection

Axonal transection was carried out using a Barkan goniotomy knife (Kaisers, Germany) on cortical cultures at 15 DIV (Blizzard et al., 2013). A single injury

extending the full diameter of the coverslip was made, ensuring complete axonal transection and producing a cell free lesion of ~50-150μm wide. Cells were fixed for 30 minutes with 4% (w/v) paraformaldehyde (PFA) in 0.01M phosphate buffered saline (PBS) at 0, 4 and 24 hours post-injury for immunocytochemistry. Sprouting response observed in cortical culture in vitro has been demonstrated to start between 3 to 6 hours post-injury (Chuckowree and Vickers, 2003). Therefore the imaging time point of 4 hours post-injury was chosen in order to match the initial sprouting response of the transected axons. The 24 hours post-injury time point was chosen to match the continued sprouting response. For electrophysiological studies coverslips were transported to the electrophysiology rig covered in culture media.

2.2.5. Immunocytochemistry

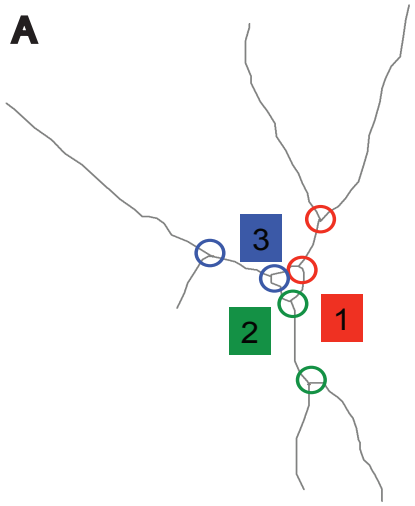
Primary antibodies used included rat monoclonal anti-green fluorescent protein to detect YFP or GFP (1:3,000; Nacalai tesque), rabbit anti-Calretinin (1:1000; Swant) and rabbit anti-activated caspase 3 (1:200, Millipore) diluted in 0.3% v/v triton-X100 (Fluka) in PBS, and applied for 1 hour at room temperature followed by overnight incubation at 4°C. Coverslips were washed thrice in PBS before the application of isotype- and species-specific secondary antibodies. Secondary antibodies [Alexa Fluor® 594- and AlexaFluor® 488-conjugated goat anti-rat and goat anti-rabbit IgG H+L (Invitrogen, USA)] were diluted 1:1000 in PBS and applied to coverslips for 2 hours at room temperature. Coverslips were washed thrice with PBS before mounting in fluorescent mounting medium (Thermo Fisher scientific, Australia).

Immunolabeling was visualized and imaged using a DMLB2 upright fluorescent microscope (Leica, Germany). For cell counts of uninjured cultures four images were taken from different regions in each coverslip. Every time point was examined in four replicate coverslips across three independent experiments. For cell counts of injured cultures and remodeling analysis, images were taken across the length of the scratch injury at increasing distances from the injury site (0-500μm from the injury, 500-1000μm from the injury site and more that 1000μm from the injury) in order to investigate distance specific effects after injury. These distances were chosen due to technical reasons representing one or two whole rotations on the X plane using a 20X magnification in a DMLB2 upright fluorescent microscope (Leica, Germany). Approximately ten images were taken from each of four replicate coverslips across three independent experiments. To further characterize the populations of *Thy1-YPF* neurons, Calretinin interneurons and *GAD67-GFP*

interneurons after injury, the cell tracing software Neurolucida™ was utilized. Each neuron was further assessed with Neurolucida Explorer to determine the dendritic angle by performing the segment dendrite analysis (Fig 2.1). For caspase immunolabeling a minimum of four images were taken from two coverslips that contained the injury site at every time point analyzed (0, 4 and 24 hours post-injury).

2.2.6. Statistical analysis

Analysis of post-injury remodeling was done using a two-way analysis of variance followed by Bonferroni post hoc tests (GraphPad Prism, version 6.0). Average values were expressed as means ± standard error of the mean. For electrophysiological comparisons of multiple groups a one-way analysis of variance followed by Bonferroni post hoc tests was used. For comparisons between 2 groups two-tailed t-tests were performed (GraphPad Prism, version 6.0). A p-value <0.05 was considered significant.



B

Tree	Order	Length(µm)	XY Angle
1	2	65.1	108.3
1	2	73.3	69.2
1	1	15.8	66.8
2	2	33.1	267.3
2	2	45.5	299.8
2	1	26.7	281.3
3	2	19.8	237.1
3	2	75.9	141.1
3	1	17.6	159.5

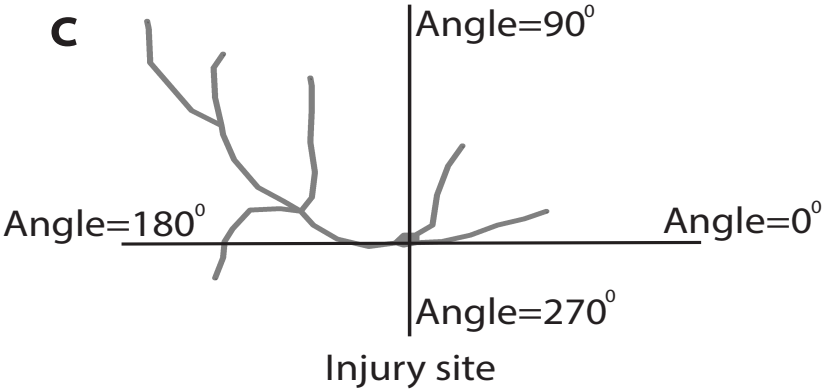


Figure 2.1 Analysis of post-injury neurite remodeling in vitro.

Representative Neurolucida tracing of CR immunolabelled cell (A) and its XY angle analysis using Neurolucida Explorer (B). Neurites were classified according to the angle they form with the injury site as projecting "away" from the injury site if they form an angle of 0 to 180 degrees with the injury site or "towards" the injury site if the form an angle of 180 to 360 degrees with the injury site (C).

2.3. RESULTS

2.3.1. Mouse cortical interneurons become functionally mature by 15 DIV

The electrophysiological characteristics of GFP^+ interneurons derived from *GAD67-GFP* transgenic mice, was performed at 7 and 15 DIV. The electrophysiological properties of these cultures were examined to determine the time required for interneurons to mature *in vitro* they had matured and express a sufficient density of voltage-gated channels to fire action potentials. Interneurons at 7 or 15 DIV were targeted for whole cell patch clamp recording, and were concurrently filled with Alexa Fluor-568 dye (Fig 2.2 A-D). Passive membrane parameters were first examined. Interneurons were classified according to their morphology *in vitro* as multipolar and bipolar. 7 DIV GFP^+ multipolar neurons had a mean resting membrane potential (RMP) of ~-59mV (n=7 cells), an input resistance of ~408MΩ, a capacitance of ~25.1pF, and a peak inward current of ~-2.01nA. By 15 DIV they became significantly hyperpolarized (~-69mV, $p<0.01$, n=11 cells) (Fig 2.2 E), their input resistance decreased from ~408MΩ to ~178MΩ (Fig 2.2 F) and their capacitance increased from ~25.1pF to ~37pF (Fig 2.2 G). These data indicated that by 15 DIV multipolar interneurons have increased in size and complexity and have begun to express significantly more ion channels at the membrane surface, potentially contributing to the observed reduction in RMP. Importantly, the peak inward current for these neurons increased from -2.01±0.4nA to -2.98±0.86nA from 7 to 15 DIV (Fig 2.2 H). Although the capacitance and I_{Na} had both increased with time in culture, these data revealed an overall increase in voltage-gated sodium channel density from 68±12 to 94±22pA/pF, which suggests that the neurons were more likely to fire action potentials.

The active membrane parameters of interneurons were also analyzed using current clamp. The presence of evoked action potentials in response to current injections has been previously reported to correlate with cell maturity (Picken Bahrey and Moody, 2003). Multipolar interneurons at 7 DIV failed to generate action potentials in response to step-wise current injections. Interneurons that had been cultured for 15 days responded to current injections by generating a train of action potentials. All multipolar interneurons fired action potentials when exposed to depolarizing current injections (Fig 2.2 M, N).

At 7 DIV *GAD67-GFP*$^+$ bipolar interneurons (n=5) had an average RMP of

~-46mV, an input resistance of ~965MΩ, a capacitance of ~13.5pF and a small inward current of ~-0.58nA (Fig 2.2 I-L). There was a significant decrease in their RMP by 15 DIV (n=20) to ~-55mV ($p<0.01$). But no significant changes in other passive membrane parameters indicating that these cells had not grown in size or complexity, or increased their voltage-gated sodium channel density during their second week *in vitro*. Although this may indicate that GFP^+ bipolar interneurons are not mature at 15 DIV, approximately 60 per cent of the neurons were able to fire action potentials. This increase in action potential frequency could likely be accounted for by their significant hyperpolarization and the slight increase in their peak inward current (n.s, $p=0.243$) by 15 DIV (Fig 2.2 M, N).

Action potential characteristics were further analyzed and compared between populations of bipolar and multipolar interneurons. Action potential amplitude and duration were used to compare the functional maturity of subpopulations of interneurons in culture at 15 DIV (Fig 2.3 A). As these properties were reported to reflect the maturity of neurons in culture (Johnson et al., 2007). There was no significant difference in action potential amplitude ($p=0.457$) or duration (half width) ($p=0.713$) between the populations of bipolar and multipolar interneurons at 15 DIV (Fig 2.3 B, C). These results suggest that, although not all bipolar interneurons were able to fire action potentials in response to depolarizing current injections, the ones that did exhibited the firing characteristics of mature neurons.

Collectively these results indicate that cortical interneurons derived from *GAD67-GFP* mice grown *in vitro* become functionally mature by 15 DIV.

2.3.2. Mouse cortical pyramidal neurons become functionally mature by 15 DIV

The electrophysiological properties of *Thy1-YFP* cultures were examined to determine whether they had matured and expressed a sufficient density of voltage-gated sodium channels to fire action potentials *in vitro*. YFP-positive neurons that were 7 or 15 DIV were used to make whole cell patch clamp recordings, and were concurrently filled with Alexa Fluor-568 dye (Fig 2.4 A-H). It was determined that YFP^+ neurons at 7 DIV had a mean resting membrane potential (RMP) of ~-65mV (n=8 cells; Fig 2.4 I), an input resistance of ~320MΩ (Fig 2.4 J), a capacitance of ~30pF (Fig 2.4 K), and a small peak inward current at 0mV of ~-1.7nA (Fig 2.4 D, L). At 15 DIV the RMP of the cultured neurons was unchanged (n=5 cells; Fig 2.4 I). However, their input resistance was reduced (Fig 2.4 J), and their capacitance

increased (Fig 2.4 K) consistent with neurons that have been maintained in culture for longer and have grown in size and complexity. Importantly, the peak inward current for neurons measured at 0mV was significantly increased from -1.7±0.3nA to -2.9±0.5nA from 7 to 15 DIV (Fig 2.4 D, H, I). Although the capacitance and I_{Na} had both increased with time in culture, these data still revealed an overall increase in voltage-gated sodium channel density from 57±9 to 72±13pA/pF, which suggested that the neurons were more likely to fire action potentials by 15 DIV.

Cortical neurons in primary culture have been reported to mature over time and elicit action potential firing patterns consistent with those described for neuronal populations in cortical slices (Mainen and Sejnowski, 1996). As the density of voltage gated sodium channels calculated above can only be used to estimate a neurons ability to fire an action potential, the firing pattern of *Thy1-YFP* labeled neurons in vitro was investigated using current clamp. At 7 DIV, neurons failed to generate an action potential in response to step-wise current injections (5 of 8 neurons did not fire an action potential, and 3 of 8 neurons fire 1 or 2 action potentials in a 200 milliseconds current step; Fig 2.4 M). Neurons that had been cultured for 15 days responded to current injection by generating a train of action potentials (all neurons examined fired in the range of 5-8 action potentials during the 200 milliseconds current injection step; Fig 2.4 N). These data indicate that cortical neurons cultured for 15 DIV better represent mature neurons *in vivo* than those cultured for 7 DIV.

2.3.3. Interneurons grown *in vitro* maintain subclass diversity

In cultures derived from *GAD67-GFP* mice GABAergic interneuron subtypes are labeled with GFP. In cultures derived from *Thy1-YFP* mice pyramidal neurons are labeled with YFP. By performing whole cell patch clamp recordings of GFP^+ and YFP^+ cells at 15 DIV the membrane properties of interneurons and pyramidal neurons were compared. During patching all neurons were filled with the Alexa Fluor-568 dye present in the internal solution, revealing their morphology (Fig 2.5 A-F). Interneurons were classified as Bipolar if they had two principal long dendrites running in opposite directions from the soma with few dendritic collaterals. Interneurons were classified as Multipolar if they present more than two dendrites emerging from the soma.

Significant differences were found in all the passive membrane parameters analyzed between the populations of multipolar and bipolar interneurons, indicating that they truly represent different and independent populations of interneurons (Fig

2.5 G-J).

There were also significant differences found in all passive membrane parameters in pyramidal neurons in comparison to bipolar interneurons (Fig 2.5 G-J). There were no significant differences found when comparing multipolar interneurons and pyramidal neurons at 15 DIV.

The electrophysiological analysis revealed at least 4 different firing patterns in response to depolarizing current injections in GFP^+ interneurons. Different firing patterns were recorded from the group of multipolar interneurons indicating that distinct subpopulations might be present (Fig 2.5 K-M). A more detailed analysis of each interneuron subclass within the multipolar group based on current clamp characteristics was attempted but was not achieved due to a limited number of recordings form interneurons in each subgroup. Only one firing pattern was recorded for bipolar interneurons (Fig 2.5 N).

These data indicate that the fundamental properties of a neuron, such as membrane potential, action potential characteristics, and ionic currents, are likely intrinsically regulated, are specific to each population of neuron and are conserved *in vitro*. These results validate the use of primary neuronal cultures as a relevant platform to investigate the response to injury of interneurons and pyramidal neurons. The *in vitro* axonal transection injury model was used in this chapter to investigate subpopulation specific post-injury alterations.

2.3.4. Calretinin interneurons remodel their neurites after injury

Neurons derived from *Thy1-YFP* transgenic mice were grown for 15 DIV, subjected to axonal transection and fixed at 0, 4 and 24 hours post-injury. Immunolabeling with antibodies to both the calcium binding protein Calretinin and YFP were used to identify Calretinin interneurons and pyramidal neurons respectively. Neurites labeled with Calretinin or YFP were traced using Neurolucida™ and a X-Y dendritic angle analysis was performed (Fig 2.6 A, B). They were then classified as being directed either “away” from the injury site if they formed an angle of 0 to 180 degrees with the injury site or “towards” the injury site if they formed an angle of 180 to 360 degrees with the injury site (Fig 2.6 C).

To quantitate injury-induced changes in the dendritic arbor orientation, Calretinin or YFP positive neurons were traced at increasing distances from the injury site and at

different time points post-injury (Fig 2.6 A-D). The orientation of individual neurites for each neuron was determined and the percentage of neurites that were orientated away or towards the injury site was calculated.

At 4 hours post-injury, for neurons located more than 500μm from the injury site, there were no significant difference in the percentages of Calretinin or YFP positive neurites that were directed away from the injury site (Fig 2.6 E, F). Further analysis indicated that for neurons located closer to the site of transection (less than 500μm) there was a significant increase in the number of Calretinin positive neurites directed away from the injury (Fig 2.6 G). The same analysis was performed at 0 and 24 hours post-injury and no significant differences were found between the two populations of neurons investigated (Fig 2.6 H, I).

To gain further insight into subpopulation specific post-injury responses, cultures derived from *GAD67-GFP* mice were grown to maturity *in vitro* and exposed to axonal injury. Neurons were fixed at 4 hours post-injury and interneurons that were either *GAD67-GFP*$^+$ or CR$^+$/*GAD67-GFP*$^+$ were investigated for their capacity to remodel after injury. Results indicate that only CR$^+$/ *GAD67-GFP*$^+$ interneurons are able to remodel their neurites away from the injury site at 4 hours post-injury at a close distance from the injury site (Fig 2.7 A, B, $p=0.005$).

In order to investigate the intrinsic functional implications of remodeling in the subpopulation of CR$^+$ *GAD67-GFP*$^+$ interneurons after injury, patch clamp recordings interneurons that usually express Calretinin was attempted. All patched bipolar *GAD67-GFP*$^+$ interneurons (at 4 hours post-injury and found at less than 500μm from the injury site) had a depolarized RMP (less than -40mV) after injury and could not be used for analysis.

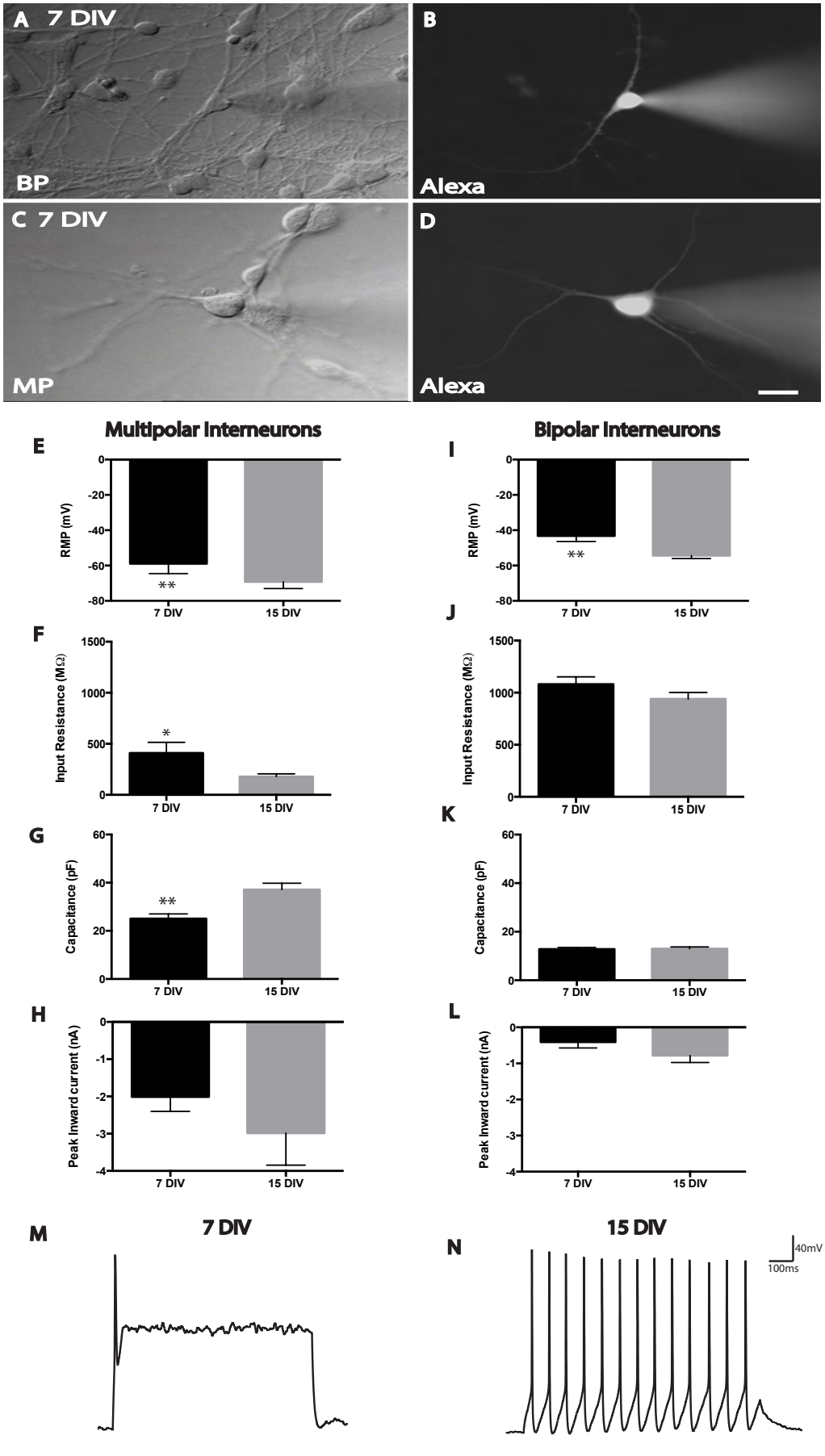

A 7 DIV
BP
B
Alexa
C 7 DIV
MP
D
Alexa
Multipolar Interneurons
Bipolar Interneurons
E
RMP (mV)
0
-20
-40
-60
-80
**
7 DIV
15 DIV
I
RMP (mV)
**
F
Input Resistance (MΩ)
1500
1000
500
0
*
J
Input Resistance (MΩ)
G
Capacitance (pF)
60
40
20
0
**
K
Capacitance (pF)
H
Peak Inward current (nA)
0
-1
-2
-3
-4
L
Peak Inward current (nA)
M
7 DIV
N
15 DIV
40mV
100ms

Figure 2.2. Development of GAD67-GFP positive interneurons in vitro.

*Phase image of GAD67-GFP positive bipolar (A) and multipolar interneurons selected for whole cell patch clamp analysis at 7 DIV (C). Bipolar (B) and multipolar (D) neurons filled with Alexa Fluor-568 dye. Passive membrane parameters of bipolar and multipolar interneurons grown for 7 and 15 DIV were analyzed. There were significant differences in RMP (-59 ± 2mV at 7 DIV and -69 ± 2mV at 15 DIV), input resistance (408 ± 106MΩ at 7 DIV and 37 ± 3 MΩ at 15 DIV) and capacitance (25 ± 2pF at 7 DIV and 37 ± 3pF at 15 DIV) in multipolar interneurons at 7 DIV (n=7) when compared with neurons grown for 15 DIV (n=11) (E, F, G). Peak inward current was measured for multipolar interneurons grown for 7 DIV (-2.1 ± 0.4nA) and 15 DIV (-2.9 ± 0.8nA) (H). RMP (-43 ± 4mV at 7 DIV and -54 ± 2mV at 15 DIV) (I), input resistance (1080 ± 123MΩ at 7 DIV and 939 ± 65MΩ at 15 DIV) (J), capacitance (13.5 ± 1pF at 7 DIV and 15 ± 2pF at 15 DIV) (K) and peak inward current (-0.58 ± 0.1nA at 7 DIV, -0.78 ± 0.2nA at 15 DIV) (L) was determined for bipolar interneurons (7 DIV (n=5), 15 DIV (n=20)). Firing pattern in response to depolarizing current injections of multipolar interneurons at 7 DIV and 15 DIV. Data are presented as mean ± SEM. Unpaired t-test *p<0.05, **p<0.01. Scale bar= 14 µm.*

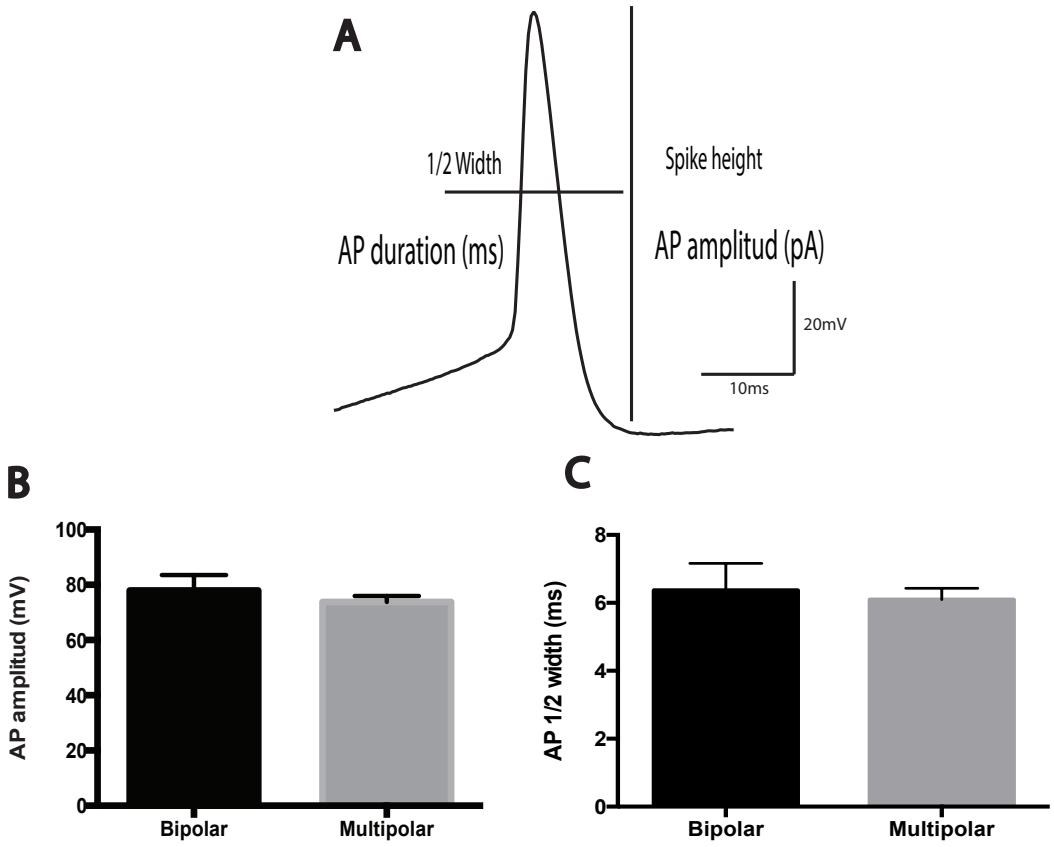


Figure 2.3. Action potential characteristics of GAD67-GFP positive interneurons in vitro.

Action potential (AP) amplitude and AP duration (AP half width) can be calculated from current clamp recordings. Action potential amplitude (B) and duration (C) were determined for bipolar and multipolar interneurons at 15 DIV. Data is presented as mean ± SEM. Unpaired t-test.

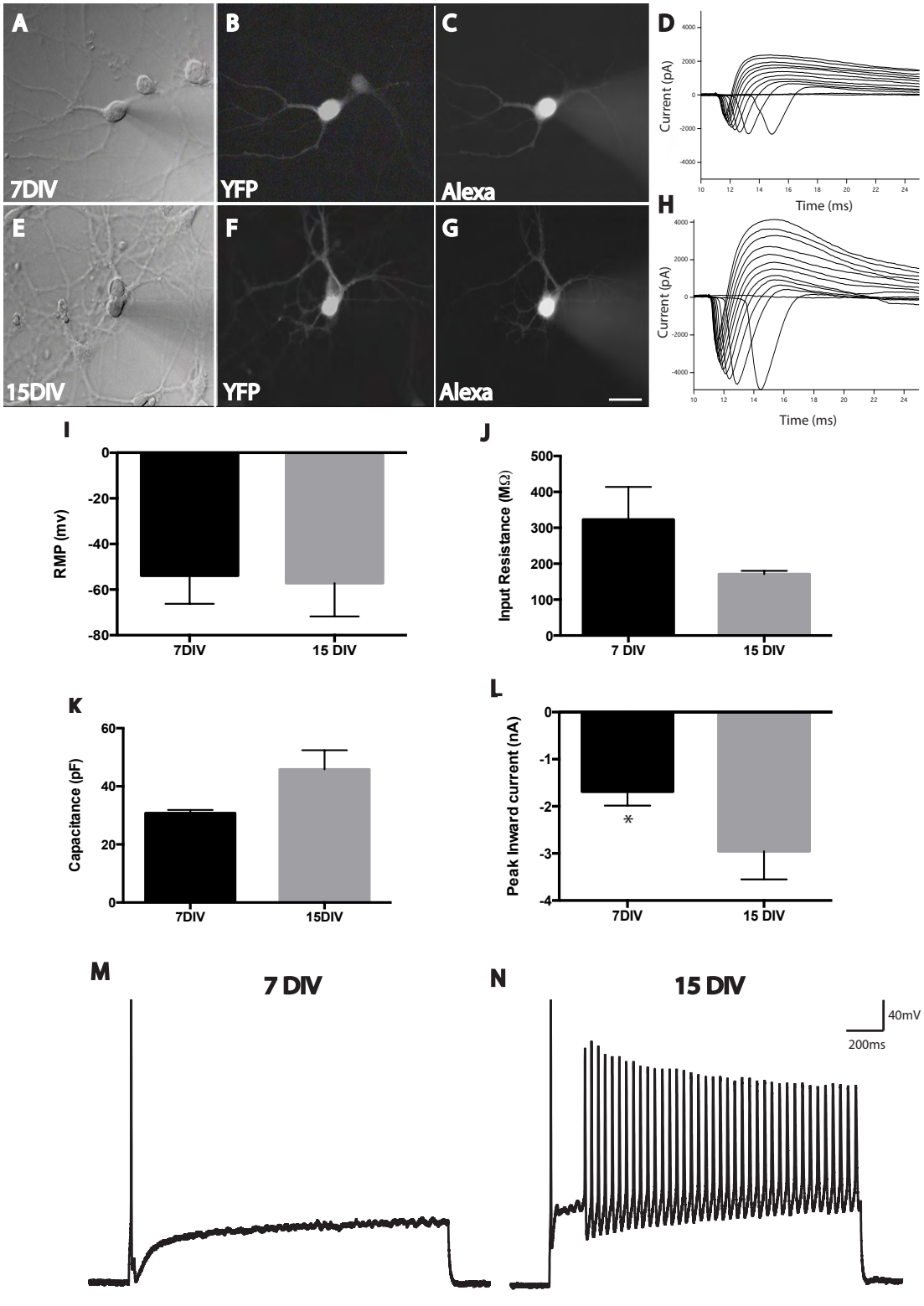
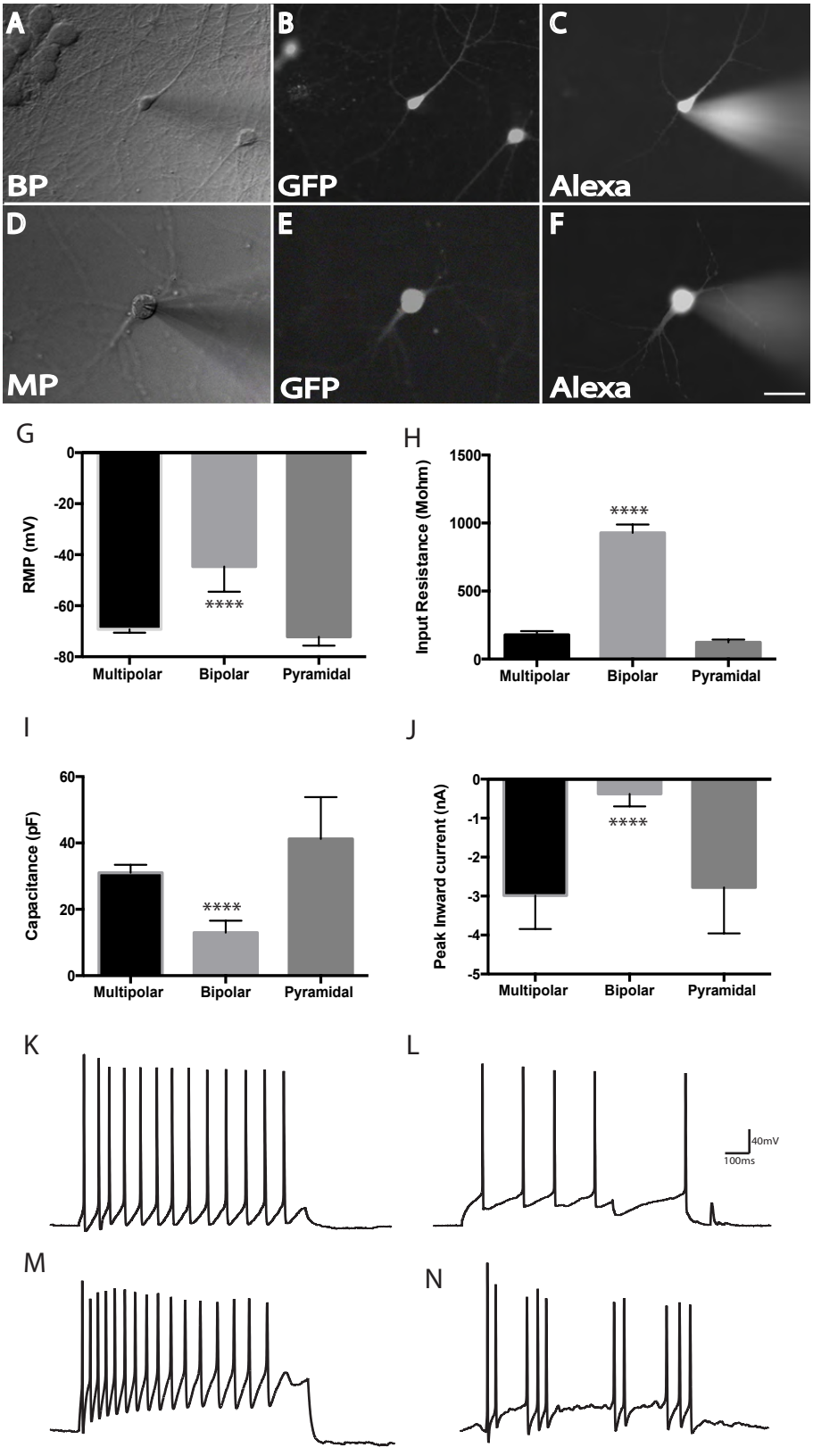

A
7DIV
B
YFP
C
Alexa
D
Current (pA)
Time (ms)
E
15DIV
F
YFP
G
Alexa
H
Current (pA)
Time (ms)
I
RMP (mv)
7DIV
15 DIV
J
Input Resistance (MΩ)
7 DIV
15 DIV
K
Capacitance (pF)
7DIV
15DIV
L
Peak Inward current (nA)
*
7DIV
15 DIV
M
7 DIV
N
15 DIV
40mV
200ms

Figure 2.4. Electrophysiological characterization of Thy1-YFP positive cortical neurons in vitro.

*Phase image of whole-cell patch clamped neurons at 7 DIV (A) and 15 DIV (E), patched neurons were YFP positive 7 DIV (B) and 15 DIV (F). Intracellular labeling of 7 DIV (C) and 15 DIV (G) neurons by Alexa Fluor-568 dye. Current responses to depolarizing voltage steps in 10mV increments from −70mV at 7 DIV (D) and 15 DIV (H). Bottom panels show bar graphs of the average electrophysiological data for YFP positive neurons at 7 DIV and 15 DIV. RMP of -65 ± 5mV (n=8 cells) at 7 DIV and -70 ± 10mV (n=5 cells) at 15DIV (I). Input resistance of 320 ± 90MΩ (n=8 cells) cells at 7 DIV and of 170 ± 9MΩ at 15 DIV (n=5 cells) (J). Capacitance of 30 ± 1pF at 7 DIV and of 44 ± 6pF (K). Peak inward current of -1.7 ± 0.3nA at 7 DIV and of -2.9 ± 0.5nA at 15 DIV on depolarization from −70mV to +50mV. Current clamp in response to depolarizing current injections in cortical neuronal cultures at 7 DIV (M) and 15 DIV (N). Unpaired t-Test *p<0.05. Data are presented as mean ± SEM. Scale bar = 14 µm.*


A
BP
B
GFP
C
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D
MP
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GFP
F
Alexa
G
RMP (mV)
0
-20
-40
-60
-80
****
Multipolar
Bipolar
Pyramidal
H
Input Resistance (Mohm)
1500
1000
500
0
****
Multipolar
Bipolar
Pyramidal
I
Capacitance (pF)
60
40
20
0
****
Multipolar
Bipolar
Pyramidal
J
Peak Inward current (nA)
0
-1
-2
-3
-4
-5
****
Multipolar
Bipolar
Pyramidal
K
L
40mV
100ms
M
N

Figure 2.5. Electrophysiological characterization of GAD67-GFP positive interneurons at 15 DIV and comparison with passive membrane parameters of Thy-1 YFP positive cortical pyramidal neurons.

*Phase image of whole-cell patch clamped bipolar (A) and multipolar interneurons at 15 DIV (D). Patched neurons were GAD67-GFP positive bipolar (B) and multipolar (E) cells. Bipolar (C) and multipolar (F) neurons are filled by Alexa Fluor-568 dye upon patching. Passive membrane parameters of bipolar (BP, n=20), multipolar interneurons (MP, n=11) and pyramidal neurons (n=8) were analyzed. RMP (G), input resistance (H), capacitance (I) and peak inward current at 0mV (J) differed between the population of bipolar and multipolar interneurons and the population of bipolar interneurons and pyramidal neurons. Current responses to depolarizing voltage steps in 10mV increments from −70mV holding potential of GAD67-GFP positive neurons grown form 15 DIV. Three firing patterns were recorded from multipolar interneurons indicating that different subpopulations of interneurons might be present in this group (K, L, M, n=3, n=5, n=3, respectively). Firing pattern in response to depolarizing current injections of bipolar interneurons (BP, n=4) (N). Data are presented as mean ± SEM. One-way ANOVA, followed by Bonferroni test ***p<0.001, ****p<0.0001. Scale bar= 14 µm.*

2.3.5. Cortical interneurons survive following *in vitro* axonal injury

As mentioned above bipolar interneurons became depolarized after injury, pointing to the possibility that they become unhealthy after injury. To investigate neuronal viability after injury, immunocytochemistry was performed at 15 DIV and at 0, 4 and 24 hours following transection injury in primary cortical cultures derived from heterozygous *GAD67–GFP* knock-in mice. Immunolabeling with antibodies to both the calcium binding protein Calretinin and GFP were used to confirm the proportion of Calretinin interneurons relative to the total number of *GAD67-GFP*$^+$ interneurons at a close distance from the injury site (at no more than 500µm from the injury site) at 0, 4 and 24 hours post-injury. The proportion of Calretinin interneurons relative to the total number of GFP$^+$ interneurons was analyzed. The number of cells in cortical neuronal cultures expressing CR increased between 9 to 12 days *in vitro,* from approximately 15 to 30 percent. The number of Calretinin interneurons reached a plateau at 15 DIV, at approximately 27 percent of GFP$^+$ interneurons. After injury there was no significant change in the number of CR$^+$ GFP$^+$ interneurons over time compared to uninjured cultures (Fig 2.8 A, B).

To further investigate the viability of Calretinin neurons “close” to the injury site, immunolabeling to detect activated Caspase 3 was performed. Caspase 3 is a protease implicated in the initiation of the neuronal cell death cascade and is therefore an important marker of cells entering the apoptotic-signaling pathway (King et al., 2013). GFP$^+$ interneurons did not express Caspase 3, indicating that they were not undergoing programmed cell death cascade (Fig 2.8 C, D). These results indicate that there was no significant apoptosis of interneurons in response to transection injury although they had become depolarized after injury.

2.3.6. YFP pyramidal neurons become hyperexcitable after injury *in vitro*

To determine changes in pyramidal neurons after injury, neurons from *Thy1-YFP* transgenic mice were grown for 15 DIV before they were subjected to axonal transection. After injury YFP$^+$ neurons were patched at increasing distances from the injury site at 4 hours post-injury and compared with YFP$^+$ neurons from uninjured cultures. Passive membrane parameters were recorded in voltage clamp and active membrane parameters in current clamp in whole cell configuration. YFP$^+$ neurons from uninjured cultures had a mean RMP of ~-72mV (n=10 cells), an input resistance of ~123MΩ, a capacitance of ~41pF, a peak inward current of ~2.8nA (Fig 2.9 C-E).

At 4 hours post-injury there were no significant changes in most passive membrane parameters. However the input resistance was altered in neurons close to the injury site (less that 500 μm) (Fig 2.9 D, p=0.009). This increase in input resistance was coupled to a hyperexcitability of neurons in injured cultures as evidenced by a significant decrease in voltage threshold (p=0.029) and significant increase in rheobase (minimal current required to fire an action potential) (p=0.008) (Fig 2.9 G, H). Furthermore, firing frequency at increasing current injections was also significantly increased in pyramidal neurons close to the injury site (p=0.035) (Fig 2.9 J).

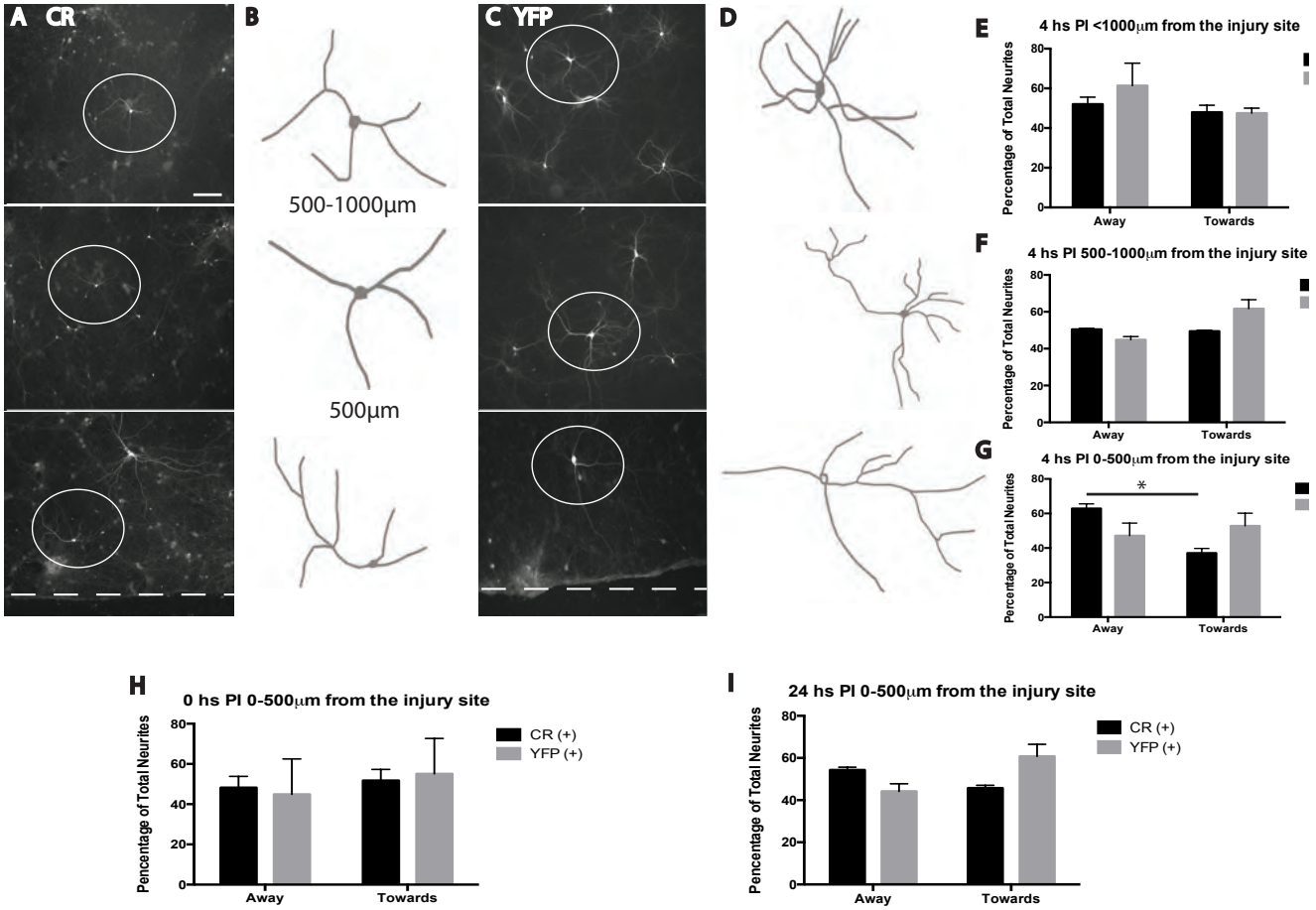


Figure 2.6. Post-injury neurite remodeling of Calretinin interneurons at 15 DIV.

Representative images and Neurolucida tracings of Calretinin interneurons and YFP pyramidal neurons at 4 hours post-injury at increasing distances from the injury site (A, B, C, D). Bar graphs represent the percentage of CR or YFP labeled neurites that were directed away or towards the injury. There was no significant angle shift found between these two populations when neurons were located further than 500μm from the injury site at 4 hours post-injury (E, F). CR interneurons located closer to the injury site (less that 500μm) remodeled away from the injury site at 4 hours post-injury (p=0.014) (G) but not at 0 or 24 hours post-injury (p=0.809, p=0.506 respectively) (H, I). Two-way ANOVA followed by Bonferroni test. Data is presented as mean ± SEM. Scale bar= 50μm.

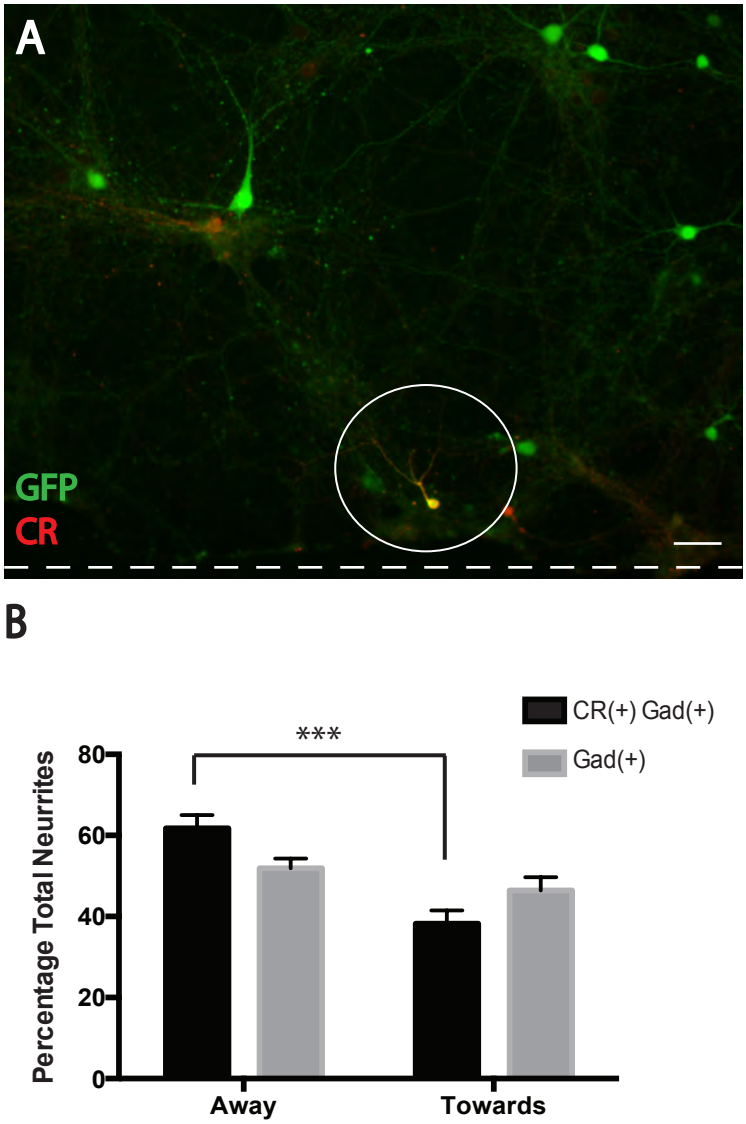


Figure 2.7. Post-injury neurite remodeling is subpopulation specific.

Representative image of CR$^+$ GFP$^+$ interneurons at 4 hours post-injury, at less than 500µm from the injury site (A). Bar graphs represent the percentage of GFP$^+$ or CR$^+$ GFP$^+$ labeled neurites that were away or towards the injury. CR$^+$ GFP$^+$ interneurons located close to the injury site (less that 500µm) remodeled away from the injury site at 4 hours post-injury (B, p=0.005). GAD67-GFP positive interneurons did not remodel away from the injury site at 4 hours post-injury. Two-way ANOVA followed by Bonferroni test. Data is presented as mean ± SEM Scale bar= 40µm.

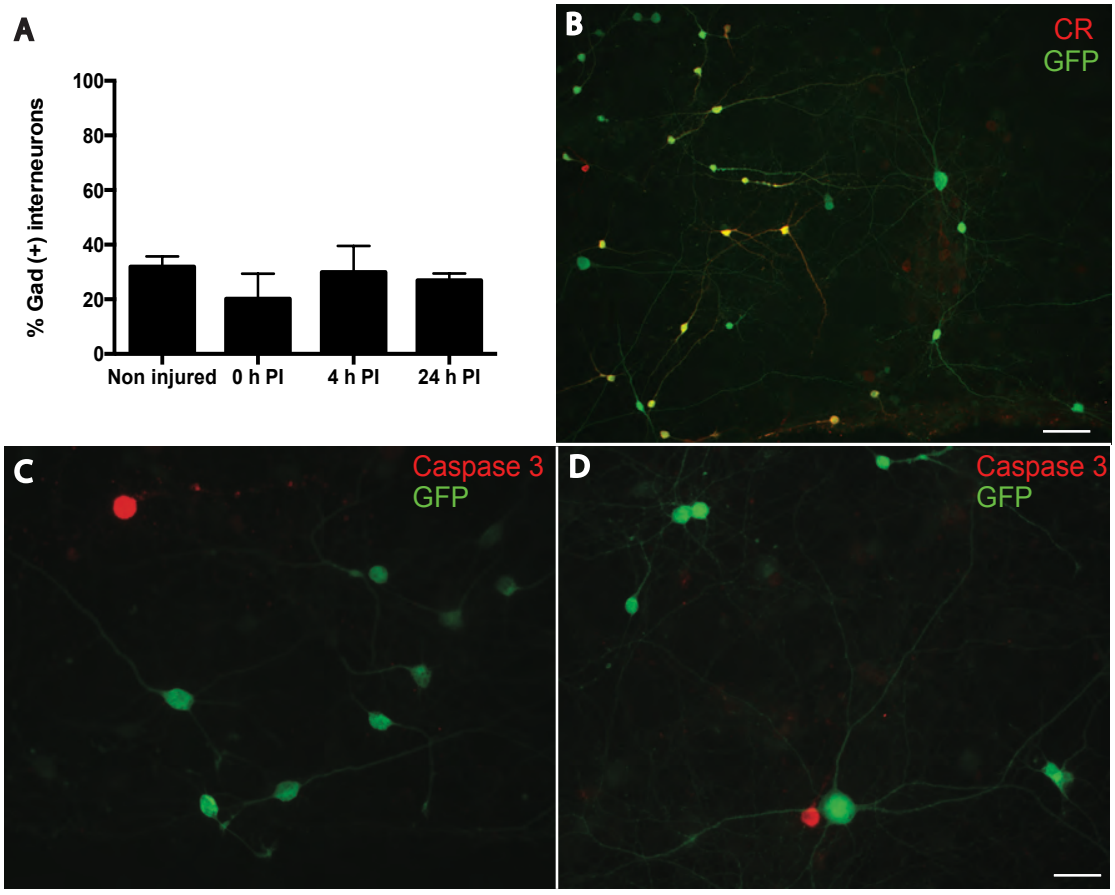


Figure 2.8. Proportion of GFP$^+$ interneurons that express Calretinin at 0, 4 and 24 hours post-injury.

There were no significant differences found in the proportion of CR$^+$ GFP$^+$ interneurons detected within the GFP$^+$ population of interneurons in uninjured or injured cultures (A) Representative image of CR$^+$ GFP$^+$ interneurons at 4 hours post-injury (B) scale bar= 50μm. Representative images of immunolabeling with anti-activated Caspase 3 antibody and anti-GFP in injured cultures at 4 hours post-injury (C, D). Data are presented as mean ± SEM Scale bar= 30μm.

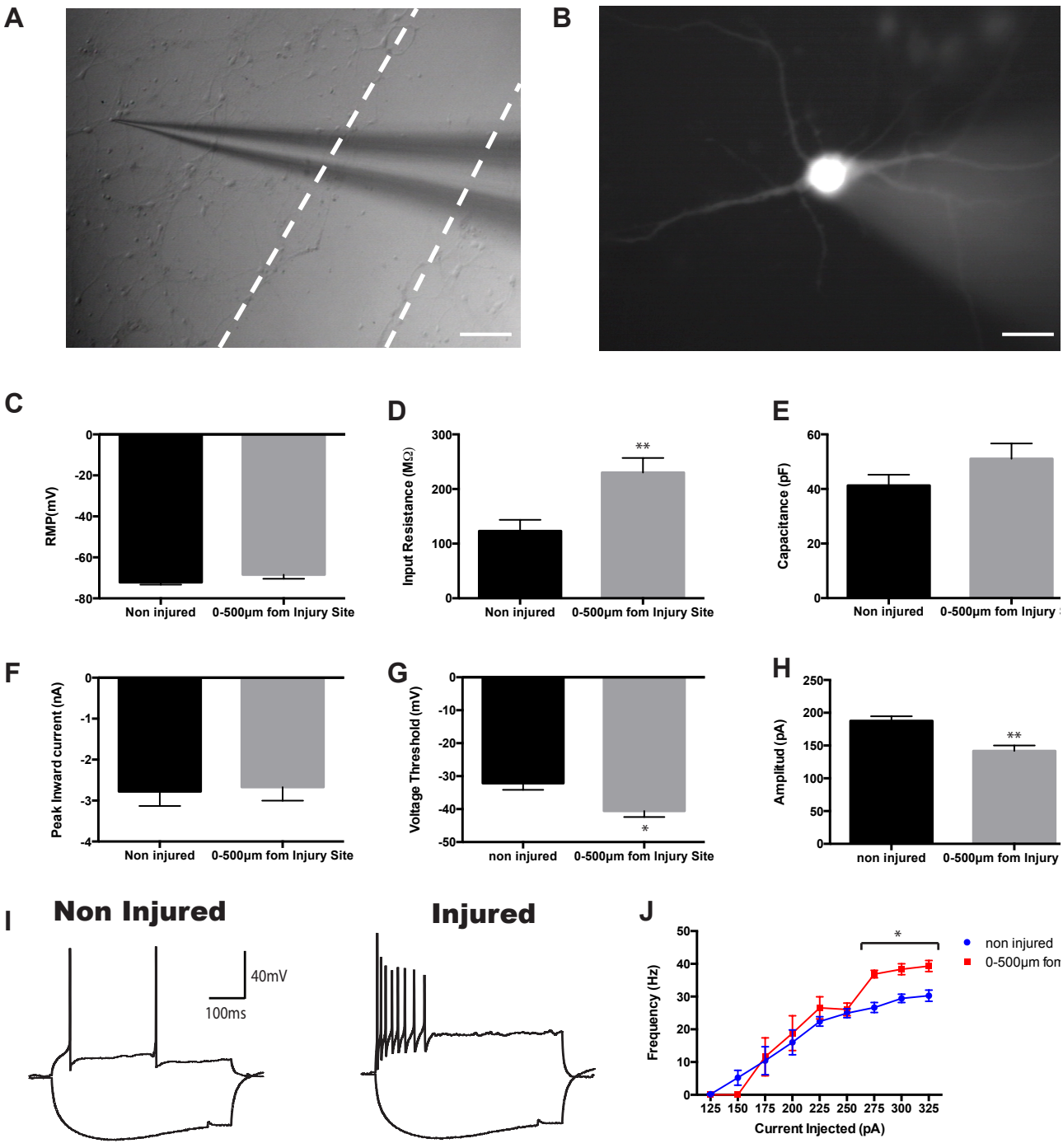


Figure 2.9. Post-injury 15 DIV Thy1-YFP positive pyramidal neurons become hyperexcitable.

Phase image of a pyramidal neuron 4 hours post-injury located less than 500µm from the injury site (A), patched neuron filled with Alexa Fluor-568 dye (B). Bar graphs represent the average electrophysiological data for YFP positive neurons in non-

*injured (n=10) and injured (n=12) cultures at 4 hours post-injury. RMP of patched YFP positive neurons in non-injured cultures was -72 ± 4mV and in injured cultures was -68 ± 2mV (C). Input resistance differed between neurons in non-injured cultures (125 ± 20MΩ) and in injured cultures (230 ± 31MΩ) (p<0.01) (D). Capacitance of neurons in non-injured cultures (42 ± 4pF) and injured cultures (51 ± 5pF) was not different (E). No differences were found in peak inward current on depolarization from −70 mV to +50 mV in neurons in non-injured cultures (-2.78 ± 0.39nA) and in injured cultures (-2.67 ± 0.31nA) (F). Voltage threshold differed between neurons in non-injured cultures (-32.6 ± 2.5mV) and neurons in injured cultures (-40.6 ± 1.8mV) (G). Rheobase also differed between neurons in non-injured cultures (187.5 ± 7.2pA) and neurons in injured cultures (141.6 ± 8.3) (H). Voltage responses to depolarizing current injections of Thy-1 YFP positive neurons at 4 hours post-injury at less than 500µm from the injury site of non-injured and injured cultures (I) Frequency-current plots of neurons in non-injured cultures and in injured cultures (J). Data are presented as mean ± SEM. Unpaired t-test *P <0.05; **P <0.01. Frequency-current plots were analyzed using a Paired-t-test *P <0.05. Scale bar 80µm (A), 14µm (B).*

2.4. DISCUSSION

In this Chapter an *in vitro* model of axonal injury was utilized to investigate the response to injury of specific subpopulations of neurons. The use of neurons derived from two transgenic mouse strains, the *Thy1-YFP* mouse and *GAD67-GFP* mouse that express fluorescent proteins in cortical pyramidal and inhibitory neurons, respectively, enabled the easy identification and study of specific neuronal populations after transection injury. First, the ability of different populations of neurons to develop and become functionally mature *in vitro* was explored. Earlier studies have indicated that the neuronal response to injury of mature and immature neurons is intrinsically different (Chuckowree and Vickers, 2003, Blizzard et al., 2007). Therefore, in the current study, an investigation of *in vitro* functional development of primary cortical cultures was carried out. Results indicated that as interneurons and pyramidal neurons matured *in vitro* there was a coordinated change in their electrophysiological properties characterized by an increase in the magnitude of voltage-gated Na^+ currents, a decrease in input resistance, an increase in capacitance and eventually, a depolarization of the resting potential that resulted in the appearance of a repetitive firing ability (Fig 2.2, 2.4).

The ability of neurons to fire a train of action potentials in response to depolarizing current injections correlates with neuronal maturity in motor neurons and cortical neurons in slice preparations (Gao and Ziskind-Conhaim, 1998, Picken Bahrey and Moody, 2003). It has also been previously reported that action potential amplitude and duration are parameters directly related to the maturity of neurons in culture (Johnson et al., 2007). These parameters were explored and used to compare the functional maturity of bipolar and multipolar subpopulations of interneurons. Results indicated that both populations of interneurons investigated had reached functional maturity by 15 DIV (Fig 2.3). Furthermore, cortical neurons in these cultures had developed firing patterns similar to those described for the interneuron population in cortical slices (Markram et al., 2004). Therefore, after the analysis of passive and active membrane parameters of interneurons grown for 2 weeks *in vitro*, it was concluded that both subpopulations of interneurons have reached maturity by 15 DIV and that neurons grown for longer periods *in vitro* better represent mature neurons *in vivo (Fig 2.5)*.

The interneuron population is remarkably diverse. To further characterize the culture model used in this Chapter, interneurons were subdivided into two major

categories: bipolar and multipolar, and significant differences in the passive membrane parameters of these populations indicated that they represent independent populations of cells. Furthermore, the action potential firing characteristics of *GAD67-GFP* interneurons revealed at least four different firing patterns. Accumulating evidence suggests that separate classes of neocortical interneurons have specific electrophysiological characteristics (Gupta et al., 2000, Kawaguchi, 2001, Markram et al., 2004, Dumitriu et al., 2007). Results in this Chapter indicate that at least four different populations of *GAD67-GFP* positive interneurons are present in culture, confirming that interneurons grown *in vitro* maintain some of the important subclass diversity found in the cortex (Fig 2.5).

Further studies in this Chapter were aimed at investigating post-injury changes in the inhibitory and excitatory neurons in culture. There is evidence indicating that interneurons are more plastic than pyramidal neurons. It has been reported before that interneurons exhibit dynamic arbor rearrangements in the adult visual cortex while pyramidal neurons remain stable (Lee et al., 2006, Lee et al., 2008). Moreover, previous experiments in the Dickson lab group have found differences in post-injury structural plasticity between Calretinin interneurons and pyramidal neurons (Blizzard et al., 2011). In the current study analysis of post-injury neurite angle indicated that CR interneurons remodel their processes away from the injury site while there were no changes found in pyramidal neurons. These results further support intrinsic differences in the neuronal response to injury between different neuronal subtypes (Fig 2.6). A possible limitation of this part of the study is the fact that analysis of dendritic angle after injury was performed in fixed samples. Ideally dendritic orientation would be investigated using live-imaging of fluorescently labeled CR positive interneurons after injury.

While there is extensive evidence indicating that inhibitory interneurons have a greater propensity to remodel, it is still unclear if all interneuron subtypes are uniform in their capacity for structural change. Previous studies indicate that in the naïve brain dendritic branch tip remodeling of interneurons in the adult visual cortex is mainly contained within a superficial strip of layer II/III of the cortex (Lee et al., 2006). Moreover it has been reported that dendritic remodeling of GABAergic interneurons is not determined by genetic lineage but rather is a general feature of all interneuron subtypes imposed only by cortical laminar circuitry (Lee et al., 2006, Lee et al., 2008, Chen et al., 2011). Although these previous elegant studies have indicated that there are no subtype specific differences in the capacity of structural

remodeling in interneurons, results in this Chapter point more to a cell type specific effect given that neurite remodeling after injury was found be restricted to CR interneurons *in vitro*. The current results seem to be contradicting previous studies, however, closer analysis reveals that this may not be the case. As mentioned above, it has been demonstrated that plastic interneurons are found in layer II/III of the cortex. Interneurons that populate layers II/III are mainly large basket cells, nest basket cells, Martinotti cells and bipolar cells. Lee at al. in 2006 reported that plastic interneurons in layer II/III in the cortex were not Pavalbumin, Somatostatin or Cholecystokinin positive. Given those results it is unlikely that Martinotti cells (usually Somatostatin positive) or Pavalbumin or Cholecystokinin positive large and nest basket cells were the plastic interneurons described in these studies. This implies that Calbindin or Calretinin positive basket cells or bipolar Calretinin cells were the interneurons defined to have a greater propensity to structural plasticity. Furthermore, it has been reported that post-injury plasticity could be specific to the Calretinin subpopulation of interneurons (Blizzard et al., 2011). In conclusion in this Chapter using an *in vitro* model of neuronal injury it was demonstrated that remodeling of dendritic arbors after injury is specific to the subpopulation of Calretinin interneurons and is not a general feature of all interneurons. This indicates that Calretinin positive interneurons may be more intrinsically plastic than other population of interneurons (Fig 2.7).

After injury there is an important release of neurotransmitters, neurotrophic factors and calcium (Weber et al., 2001, Morrison et al., 2011). Given that remodeling was found to be restricted to an area close to the injury site and at an early post-injury time point, it is plausible that the release of these diffusible factors around an injury site might have differential effects on specific cell types mediating neurite remodeling on Calretinin interneurons. Although this would need to be further investigated.

To gain insights into the functional consequences of dendritic remodeling, post-injury patching of Calretinin interneurons was attempted. Bipolar interneurons were targeted since they typically express Calretinin (Markram et al., 2004). Post-injury patching of bipolar interneurons proved to be technically very challenging and was not successful. As a balance of excitation and inhibition is critical for normal cortical function it was hypothesized that the alterations in the dendritic arbor of cortical interneurons might produce an imbalance in cortical excitation. In order to test this hypothesis post-injury patch-clamp of *Thy-1 YFP* positive pyramidal neurons

was performed. Results indicated that pyramidal neurons had become hyperexcitable presenting an increased firing frequency four hours after injury (Fig 2.9). Further analysis of electrophysiological parameters demonstrated that these cells had an increased input resistance. An increase in input resistance has been previously reported in *in vitro* models of partially isolated cortex, the undercut injury model and also in *in vivo* models of the chronically injured epileptogenic cortex and has been linked to an increased firing frequency (Prince and Tseng, 1993, Jin et al., 2005, Jin et al., 2006). Furthermore, an increase in input resistant would indicate that less current would be necessary to excite these cells and this was demonstrated by a decrease in rheobase. Moreover, YFP^+ neurons in injured cultures close to the injury site presented a significant increase in voltage threshold and a steeper relationship between action potential frequency and depolarizing current. There were no differences found in RMP, peak inward current or capacitance suggesting that the overall size and complexity of the neurons in injured and uninjured cultures was similar and therefore suggesting that neurons that were patched had not been directly injured (Fig 2.9).

Neuronal excitability can be influenced by afferent synaptic excitatory and inhibitory balance as well as an intrinsic excitability of the neuron, which in turn is mainly determined by its membrane proteins and their activity (Smith et al., 2015). The differences found in the electrophysiological parameters in pyramidal neurons after injury in the current study would point to an increase in intrinsic excitability of these neurons. Several mechanisms can be proposed as responsible for this effect. Firstly, the increased intrinsic excitability found could be related to the initiation of the sprouting response present at 4 hours post-injury given that maladaptive axonal sprouting of excitatory neurons has been previously linked to hyperexcitability (Prince et al., 1997, Prince et al., 2009). Secondly, it is plausible that the presence of diffusible factors released after injury like excitatory neurotransmitters that might act via metabotropic receptors are responsible for the increased excitability of pyramidal neurons. Thirdly, it is also likely that a decrease in inhibitory input onto pyramidal neurons, due to interneuron remodeling, could drive the increased excitability found. Calretinin interneurons have been described to be mainly interneuron selective inhibitory cells that specifically target dendritic inhibitory interneurons (Gulyas et al., 1996, Caputi et al., 2009). The fact that they target almost exclusively other subtypes of interneurons indicates that even though Calretinin interneurons represent only 20 percent of the total interneurons in a culture, changes in this subpopulation could have larger downstream effects than the expected due to their number. It is therefore

hypothesized that neurite remodeling in Calretinin interneurons could have an important impact in the whole inhibitory circuit. Since they target almost exclusively other subtypes of interneurons it is hypothesized that their neurite remodeling could have larger downstream effects than at first expected due to their proportion in the total population of interneurons. In support of this hypothesis remodeling of Calretinin interneurons and hyperexcitability of pyramidal neurons were found in the same region of the injured coverslip and at the same post-injury time point. Thus is likely that Calretinin interneuron neurite remodeling, post-injury axonal sprouting and the presence of diffusible factors release form the injury site are all contributing factors in the imbalance of excitation and inhibition linked to the development of post-traumatic hyperexcitability in this model.

Primary cortical neuronal cultures can replicate many specific features of the CNS and allow the study of key neuronal changes in a simple cellular environment that is more amenable to experimental manipulation. In the current study the use of primary neuronal cultures from cells derived from knock-in transgenic mice allowed the analysis of excitatory and inhibitory neurons after injury. Results in this Chapter build on previous evidence indicating that there is a differential response to injury of excitatory and inhibitory neurons specifically Calretinin interneurons. Furthermore, the results indicate that there is a possible link between post-injury plasticity of inhibitory neurons and altered excitability. Additional studies presented in Chapter 3 investigating inhibitory synaptic input onto pyramidal neurons, further contribute to a better understanding of the mechanisms involved in increased excitability after injury.

3. MILD TRAUMATIC INJURY INDUCES MORPHOLOGICAL CHANGES IN CALRETININ INTERNEURONS AND REDUCES GABAERGIC INHIBITION IN THE CORTEX

3.1 INTRODUCTION

Mild injuries account for approximately 80 percent of the total number of brain injuries that occur globally (Roozenbeek et al., 2013). After a mild injury to the brain most patients do not usually display any clear morphological or functional abnormalities (Bigler and Maxwell, 2012). While most patients recover completely from a mild brain injury, up to a quarter can have persistent cognitive, behavioral and emotional complaints and a small proportion can develop post-traumatic epilepsy (McAllister et al., 2006, Kennedy et al., 2007, Xydakis et al., 2008, Vasterling et al., 2009, Combs et al., 2015). The development of these clinical complications may be attributed to the activation of plastic adaptive and maladaptive processes after injury. While adaptive changes potentially lead to recovery, maladaptive ones can result in injury-induced reorganization of neuronal networks that may worsen outcomes after injury. The specific mechanisms involved in the functional alterations after injury remain poorly understood and elucidating these mechanisms continues to be a fundamental priority in neuroscience research.

Traumatic injuries are often followed by alterations in the excitability of surviving cortical networks. The role of glutamate signaling in the pathology of TBI in particular has been well described. There is vast evidence indicating that acute post-traumatic glutamate release is related to excitotoxicity that leads to neuronal injury, death and dysfunction; and that delayed disruption of glutamate circuits leads to further cognitive and motor deficits after TBI (Guerriero et al., 2015). It is assumed that the perturbations in the glutamate circuit finally result in an imbalance of excitatory and inhibitory synaptic transmission. There is considerable evidence from animal studies that mild TBI induce pathology in the hippocampus and the thalamus, where disruption of the necessary balance between excitation and inhibition can be linked to post-traumatic epilepsy and cognitive impairment (Hunt et al., 2011, Pavlov et al., 2011, Huusko and Pitkanen, 2014, Almeida-Suhett et al., 2015, Drexel et al., 2015, Girgis et al., 2016). Modifications in the inhibitory network have been well described in the hippocampus with reports indicating that mild TBI causes alterations in the number of GABAergic interneurons and changes in the expression of various GABA receptor subunits, leading to a reduction in inhibitory and increase in excitatory post-synaptic currents (Hunt et al., 2011, Almeida-Suhett et al., 2015, Drexel et al., 2015).

Altered neurotransmission has also been found in other regions of the brain after mild TBI, for example the basolateral amygdala where it has been linked to post-traumatic anxiety (Almeida-Suhett et al., 2014).

Recent evidence indicates that the normal balance between excitatory and inhibitory neurotransmission is also disrupted in the cerebral cortex after mild TBI (Ding et al., 2011, Smith et al., 2015, Ping and Jin, 2016). Intrinsic electrophysiological changes were detected in layer 5 pyramidal neurons, as well as increased excitability (Greer et al., 2012, Hanell et al., 2015b). As mentioned before while there is accumulating evidence of an increased excitatory drive in the cortex after mild injury, there is still a lack of information regarding pathogenic changes in inhibitory circuits following injury to the brain. Thus the aim of this Chapter was to further investigate plastic alterations in the cortical inhibitory network after mild injury to the brain using a clinically relevant *in vivo* model of focal and diffuse injury. Morphological studies into specific subpopulations of interneurons were performed in order to build on the *in vitro* observations presented in Chapter 2.

3.2. MATERIALS AND METHODS

3.2.1. Fluid percussion injury model

All experiments were approved by the Animal Ethics Committee of the University of Tasmania and performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (A0014233). Animals were housed in individually ventilated cages at 20°C, on a 12 hour light-dark cycle, with access to food and water *ad libitum*. Male transgenic *Thy1-YFP-H or GAD67-GFP* mice were utilized in this study. For immunohistochemical labeling and analysis only *GAD67-GFP* mice were used.

Adult animals were exposed to mild TBI at 8-10 weeks of age in the form of a lateral fluid percussion injury (FPI). Animals were anesthetized with isoflurane and placed in a stereotaxic frame, the scalp was opened, and the fascia scraped from the skull. Using a trephine, a 3 mm diameter craniectomy was performed over the somatosensory cortex and a needle hub (3 mm inner diameter) was secured above the skull opening. Post craniectomy (1-2 hours later) animals were anesthetized with isoflurane anesthesia. Once surgical anesthesia was achieved the isoflorane was withdrawn, the hub was filled with saline and mice were connected to the fluid percussion injury device. Once a normal breathing pattern resumed, but before sensation was restored, an injury was induced by releasing the pendulum onto a fluid-filled piston which induced a brief fluid pressure pulse upon the intact dura (1.4 ± 0.10 atmospheres). Animals were allowed to partially recovery from anaesthesia before injury since the righting time (the time taken for the mouse to regain its righting reflex) was used as a measure of brain injury. After injury the hub was removed and the animal was placed under isoflurane for suturing. Sham animals received the same surgeries, without receiving the injury.

To identify cells generated in the post-injury period, the thymidine analogue 5-ethynyl-2′-deoxyuridine (EdU, 0.2mg/ml; Invitrogen), was administered *ad libitum* via the drinking water, as previously described (Clarke et al., 2012). Delivering EdU in the drinking water is a standard method for tracing newly generated cells over longer periods of time (Clarke et al., 2012, Wang et al., 2016). EdU is incorporated into the DNA of dividing cells during S phase of the cell cycle. Mice were sacrificed at 1 day, 1 week and 4 weeks post-injury. Mice (n=6 FPI injured and n=6 sham injured per time point) were terminally anaesthetized (sodium pentobarbitone, 140mg/kg, i.p.)

and transcardially perfused with 4% paraformaldehyde in PBS. Brains were post-fixed in 4% PFA-PBS overnight and stored at 4°C in PBS containing 0.1% w/v sodium azide (Sigma Aldrich, Australia).

For sectioning brains were embedded in 5% (w/v) molecular grade agarose (Bioline Australia Pty Ltd, NSW Australia) dissolved in PBS. Coronal sections (50µm thick) were produced using a Leica VT1000S vibratome (Biosystems, Australia), and collected as floating sections into 24 well plates (Corning Life Sciences, USA) containing PBS sodium azide.

3.2.2. Immunohistochemistry and EdU detection

Free-floating sections were stored in darkness at 4°C until processed for immunohistochemistry. The coronal brain sections were processed using standard immunohistochemical methods. Somatosensory cortices containing the injury site were identified by referring to anatomical landmarks such as the appearance of the lateral ventricles, the third ventricle and the corpus callosum. Sections were incubated for 24 hours at room temperature with primary antibodies that included, rat anti-GFP protein to detect YFP or GFP (1:3,000; Nacalai tesque) and rabbit anti-CR (1:1000; Swant) in PBS containing 0.3% Triton X-100. Sections were then washed three times in PBS and incubated with isotype- and species-specific Alexa Fluor-488 or -568; conjugated donkey anti-rabbit (1:1000) or goat anti-rat (1:1000) (Thermo-Fisher Scientific, Australia) for two hours at room temperature. After the final immunohistochemistry washes EdU was visualized using the AlexaFluor-647 Click-iT EdU kit (Life Technologies, Australia), as previously described (Young et al., 2013). Sections lacking primary antibodies were prepared to control for any non-specific binding of secondary antibodies.

Quantification of the total number of interneurons was based on the endogenous expression of GFP enhanced by the use of an anti-GFP antibody on *GAD67-GFP* transgenic mice. Quantification of the subpopulation of Calretinin interneurons was based on their expression of this calcium binding protein. Interneuron numbers were quantified in the injury site in the supragranular (layers I-IV) and infragranular (layers V-VI) lamina in 6 age-matched sham and 6 FPI mice per time point. Images were collected using an UltraVIEW spinning disk confocal microscope running Volocity Software (PerkinElmer, Australia) equipped with a Plan Apo 60x/1.20 water objective (Nikon, USA) configured to capture 30µm Z-stacks

(slices 1µm apart). The supragranular and infragranular lamina were defined as two adjacent fields containing cortical layers I-IV and V-VI, respectively, and demarcated as extending from the pial surface to the grey-white matter border. To perform unbiased and inclusive quantitative analysis, ImageJ freeware was utilized for automation of cell counts.

Neuronal morphology was characterized using Neurolucida^{TM} software. Calretinin positive cells were assessed for alterations to Calretinin-labeled neurites (including axons and dendrites). Tracing was performed in 30µm Z-stacks (slices 1µm apart) to allow for accurate reconstruction of neurons and avoid background fluorescence or overlapping of Calretinin positive processes.

3.2.3. Electrophysiological characterization of neurons *in vivo*

Thy1-YFPH or *GAD67-GFP* mice were exposed to either FPI or sham injuries at 8 weeks of age. Following cervical dislocation, mice were decapitated and their brains dissected into ice-cold sucrose solution containing: 75mM sucrose, 87mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4, 25mM $NaHCO_3$, 7mM $MgCl_2$, and 0.95mM $CaCl_2$. Acute coronal slices (300 µm) were prepared using a Leica VT1200s vibratome, and incubated at 31.7°C for ≥ 45 minutes in artificial cerebral spinal fluid (ACSF) containing: 119mM NaCl, 1.6mM KCl, 1mM NaH_2PO_4, 26.2mM $NaHCO_3$, 1.4mM $MgCl_2$, 2.4mM $CaCl_2$, and 11mM glucose (300 ± 5 mosm/kg). During recordings, slices were perfused with ACSF containing 500nM tetrototoxin and 1mM kynurenic acid, saturated with 95% O_2 / 5% CO_2 (all sigma). Whole cell recordings were obtained from layer V pyramidal cells just below the point of injury, identified by the shape of the cell soma and presence of a long apical dendrite. Neurobiotin (1.5 mg/ml) was included in the intracellular solution and slices were recovered for histological processing. Recording electrodes were prepared from glass capillaries and had a resistance of 3-5 MΩ when filled with an internal solution containing 120mM cs-methanesulfonate, 20mM HEPES, 0.4mM EGTA, 5mM TEA, 2mM $MgCl_2$, 2.5mM MgATP, 0.3mM GTP and set to a pH of 7.2-7.3 and an osmolarity of 280 ± 5 mOsm/kg. Recordings were collected using a HEKA patch clamp EPC800 amplifier, with a holding potential of 0 mV. Recordings were made using a gap free protocol for 3 minutes and 10 seconds, sampled at 50kHz and filtered at 1kHz using PClamp10 software (Molecular Devices). Data was not included in the analysis if the access resistance was ≥ 20 MΩ, or if the access resistance changed by ≥ 10% during the course of the recording. Data collection was carried out only if the recordings were

stable for more than 3 minutes. Mini inhibitory post-synaptic currents (mIPSCs) were defined as having an amplitude ≥ 8 pA, and were analysed using the MiniAnalysis60 program (Synaptosoft, USA). Post-recording, the patch electrode was removed from the neuron, which was later identified by the detection of neurobiotin with an Alexa Fluor 594 conjugated anti-streptavidin antibody (Molecular Probes™; 0.1% triton-X-diluent detergent), to confirm that recorded cells were indeed layer V pyramidal neurons in the injury site.

3.2.4. Statistical analysis

All data was analyzed using a two-way analysis of variance followed by Bonferroni post hoc tests (GraphPad Prism, version 6.0) for group and regional comparisons. Average values were expressed as means ± standard error of the mean. For electrophysiological comparisons, two-tailed t-tests were performed. A *p*-value <0.05 was considered significant.

3.3. RESULTS

3.3.1. Mild TBI does not induce cortical loss or contusion

Mild brain injury was induced in *GAD67-GFP* mice using the lateral fluid percussion injury model. Mild injury to the brain was confirmed by a short post-injury righting time (between 2-4 minutes) and no mortality after injury (Table 1) (Alder et al., 2011). Mild FPI induced a transient suppression of the righting reflex in all injured animals of 2.60 ± 0.58 min that was significantly longer than that of 0.15 ± 0.10 min for sham injured animals ($p<0.001$). Furthermore, no significant difference in righting reflex suppression was noted between injury groups of 1 day, 7 days and 28 days post-injury. Indicating that all groups received injuries of comparable severity. Moreover, there were no significant differences in age and weight between the sham operated and FPI injured mice or between time groups. A transient apnea was observed in several animals following injury (Table 1). This apneic episode was usually of short duration (3-5 seconds) and resolved spontaneously without intervention.

After lateral fluid percussion injury, qualitative histological observation of the brain revealed that mild injury to *GAD67-GFP* mice does not result in contusion, intraparenchymal hemorrhage or cortical loss (Fig 3.1).

3.3.2. Mild TBI does not lead to interneuron loss

In order to determine whether mild TBI had an effect on the population of interneurons, the total number of GFP positive interneurons was examined in *GAD67-GFP* mice at 1 day, 7 days and 28 days post-injury. Coronal cortical sections from sham and FPI injured mice ipsilateral and contralateral to the injury site were immunostained to detect GFP (Fig 3.2 A, B; 7 days PI). The total number of GFP positive interneurons was quantified, revealing that mild brain injury does not affect the total number of interneurons present in the ipsilateral or contralateral somatosensory cortex at 1, 7 or 28 days post-injury (Fig 3.2 C; $p=0.85$). As subpopulations of interneurons populate specific cortical layers, the total number of interneurons in different layers of the cortex was also analyzed. There was no significant influence of injury on the number of interneurons present in the supragranular (Fig 3.2 D; layers I-IV; $p=0.77$) or infragranular layers (Fig 3.2 E; layers V, VI; $p=0.66$). These results indicate that there were no regional specific changes in

	1 day PI		7 days PI		28 days PI	
	Sham n=6	FPI n=6	Sham n=6	FPI n=6	Sham n=6	FPI n=6
Age (weeks)	11.3 ± 0.8	9.6 ± 0.7	11.2 ± 0.5	11.3 ± 0.6	12.7 ± 0.2	12.5 ± 0.5
Weight (g)	28.6 ± 0.9	27.3 ± 1.4	29.2 ± 0.6	29.9 ± 0.9	26.0 ± 0.8	26.2 ± 1.1
FPI (atm)	NA	1.44 ± 0.10	NA	1.37 ± 0.12	NA	1.39 ± 0.15
Righting time (min)	0.2± 0.1	2.16 ± 0.53**	0.1± 0.1	2.67 ± 0.61**	0.1 ± 0.1	2.04 ± 0.30**
Apnea (sec)	NA	2.6 ± 0.8	NA	4.1 ± 0.5	NA	3.7 ± 0.8

Table 3.1. Summary of age, weight, fluid pulse pressure delivered and the righting time of sham or FPI GAD67-GFP mice.

*Injured animals had an increased righting time when compared to sham animals. There were no differences in age or weight between groups. There were no differences in the pulse of pressure received by injured groups. Data are presented as mean ± SEM. Two-way ANOVA, followed by Bonferroni test (**p<0.01).*

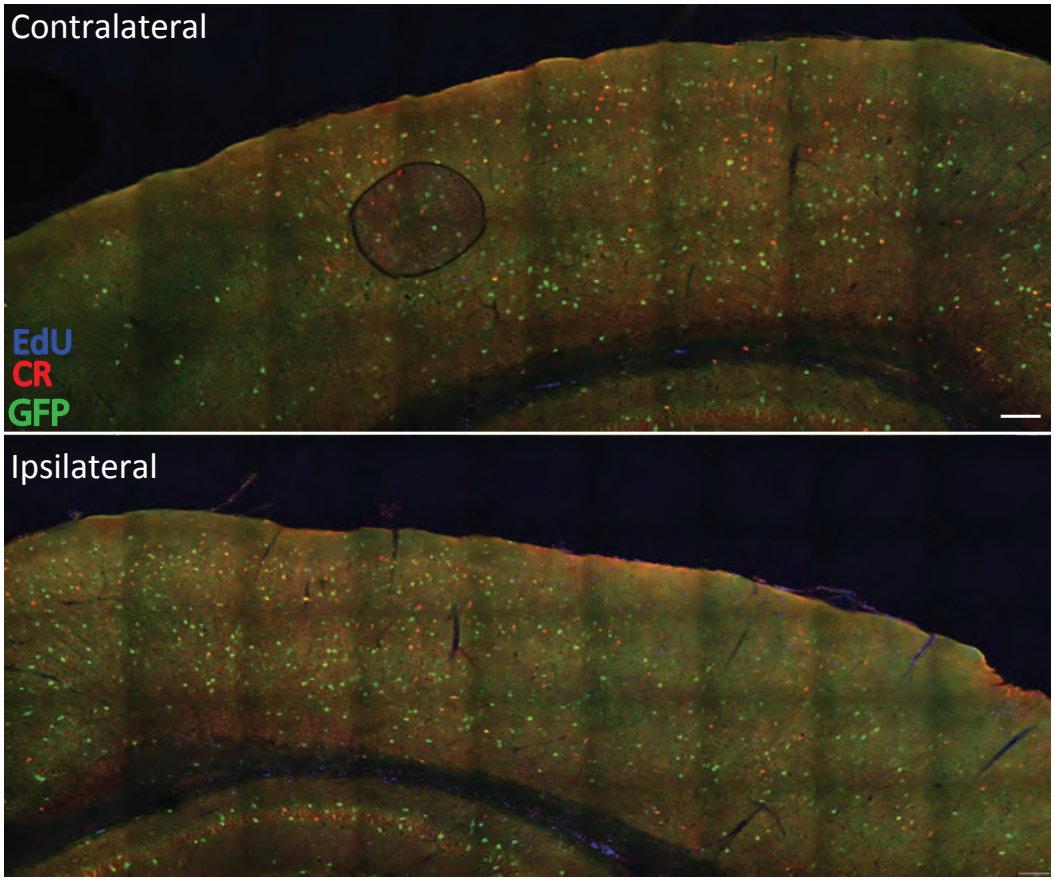


Figure 3.1. Representative image of post-injury cortical tissue from a GAD67-GFP mouse containing the injury site.

Qualitative observation of post-injury cortical tissue indicated that there was no clear cortical loss after injury at any of the time point post injury analyzed, however quantitative analysis was not within the scope of this thesis. *Immunolabeling for Calretinin (CR), EdU and GFP contralateral and ipsilateral to the injury site after FPI. Scale bar=100μm*

particular subpopulations of interneurons after mild TBI.

3.3.3. Calretinin interneurons survive for at least 4 weeks following a mild TBI

Previous results in Chapter 2 point to Calretinin interneurons as an important population of interneurons with a unique response to injury. To build on the earlier results the population of Calretinin interneurons was investigated after mild TBI in *GAD67-GFP* mice at 1, 7 and 28 days post-injury. Coronal cortical sections from sham and FPI mice were immunostained with anti-Calretinin antibody to detect Calretinin positive interneurons (Fig 3.3 A, B; 7 days PI). The total number of Calretinin positive interneurons was quantified ipsilateral and contralateral to the injury site. Mild brain injury had no effect on the total number of Calretinin interneurons ipsilateral or contralateral to the injury site at any of the post-injury time points analyzed when compared to sham mice (Fig 3.3 C; $p=0.32$).

Calretinin expressing interneurons include bipolar cells (layers II-VI), double bouquet cells (layers II-VI) and bitufted cells (preferentially located in layers I-IV) (Markram et al., 2004). To further investigate possible injury effects on specific subpopulations of Calretinin interneurons the number of Calretinin interneurons in different layers of the cortex was analyzed. There were no significant differences found in total number of Calretinin interneurons in the supragranular (Fig 3.3 D; layers I-IV; $p=0.55$) or infragranular layers (Fig 3.3 E; layers V, VI; $p=0.50$) of the cortex at any of the post-injury time points examined. These results further indicate that mild TBI does not lead to the loss of specific subpopulations of interneurons.

3.3.4. Mild TBI induces cell proliferation but not neurogenesis

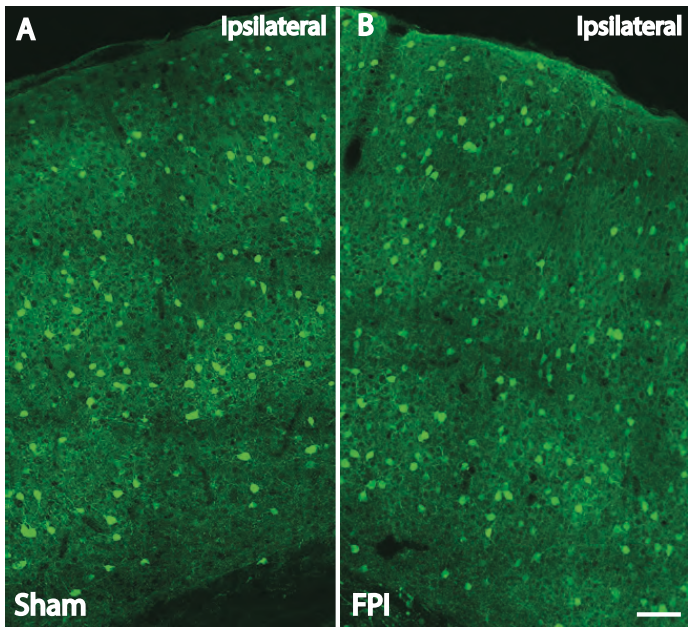
In order to investigate the potential induction of neurogenesis after injury, EdU was administered via the drinking water to sham and FPI mice for 1, 7 or 28 days post-injury. The number of newly generated cells that were EdU labeled was quantified in the ipsilateral and contralateral cortex of sham and FPI mice (Fig 3.4 A, B). There was no significant increase in the number of EdU labeled cells in the somatosensory cortex at 1 day post-injury, ipsilateral or contralateral to the injury site when comparing sham or FPI mice. However the number of EdU positive cells detected at 7 days post-injury was significantly increased in the ipsilateral cortex of FPI and sham operated mice compared to their contralateral cortices. There was

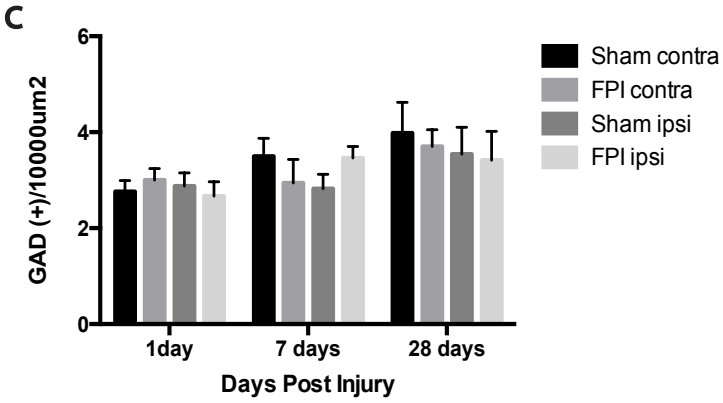
also a significant increase in the number of EdU positive cells in FPI mice relative to sham mice ipsilateral to the injury site at 7 days post-injury. EdU immunopositive nuclei were rarely observed in tissue sections contralateral to the injury site. The increase in EdU positive cells was maintained in the ipsilateral cortex of Sham and FPI animals by 28 days post-injury indicating that there was no death of labeled cells at this later post-injury time points (Fig 3.4, E, F).

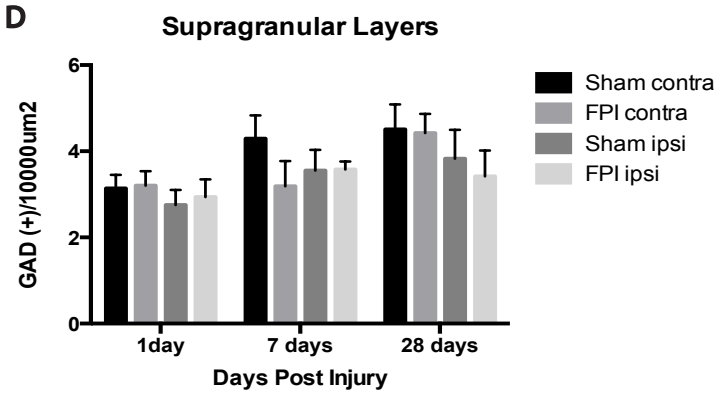
To determine if mild TBI induced interneuron generation or Calretinin interneuron production the presence of GFP^+ EdU^+ and of CR^+ EdU^+ interneurons was evaluated. There were no newly generated GFP^+ interneurons or CR^+ interneurons detected in the injured cortex ipsilateral or contralateral to the injury site in the FPI or sham animals at any of the post-injury time points analyzed (0 GFP^+ EdU^+ cells from a minimum of 200 GFP^+ cells per section per animal and 0 CR^+ EdU^+ cells from a minimum of 25 CR^+ cells per section per animal) (Fig 3.4 G, H).

3.3.5. Mild TBI induces morphological changes in Calretinin interneurons

Results in Chapter 2 indicate that Calretinin interneurons respond to transection injury by rearranging their dendritic arbors away from the injury site. In order to determine if Calretinin interneurons respond to mild diffuse and focal injury to the brain in a similar fashion, Calretinin interneurons found in the injury site and contralateral to the injury site at 7 and 28 days post-injury were examined and their morphology was traced from confocal stacks using Neurolucida software. After tracing and reconstruction of interneurons, parameters such as mean dendritic length, soma size and branch order and number were calculated using Neurolucida Explorer. At 7 days post-injury it was determined that the total length of the dendritic arbor of Calretinin interneurons was reduced in the ipsilateral side of FPI mice relative to sham control animals (64 neurons were analyzed per mouse for n=6 mice per group, two-way ANOVA, $p=0.004$). However this effect was not maintained in the long-term as total dendritic length was equivalent for Calretinin interneurons in the ipsilaretal cortex of sham and FPI mice by 28 days post-injury (Fig 3.5 B, C). No morphological differences were detected in neurons on the contralateral side of sham or PFI injured mice at 7 or 28 days post-injury (Fig 3.5 A, B, C, Two-way ANOVA, $p=0.22$).


A
Ipsilateral
Sham
B
Ipsilateral
FPI


C
GAD (+)/10000um2
0
2
4
6
1day
7 days
28 days
Days Post Injury
Sham contra
FPI contra
Sham ipsi
FPI ipsi


D
Supragranular Layers
GAD (+)/10000um2
0
2
4
6
1day
7 days
28 days
Days Post Injury
Sham contra
FPI contra
Sham ipsi
FPI ipsi

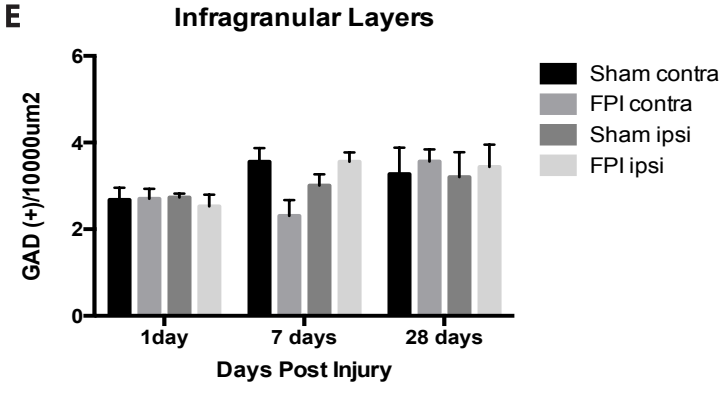
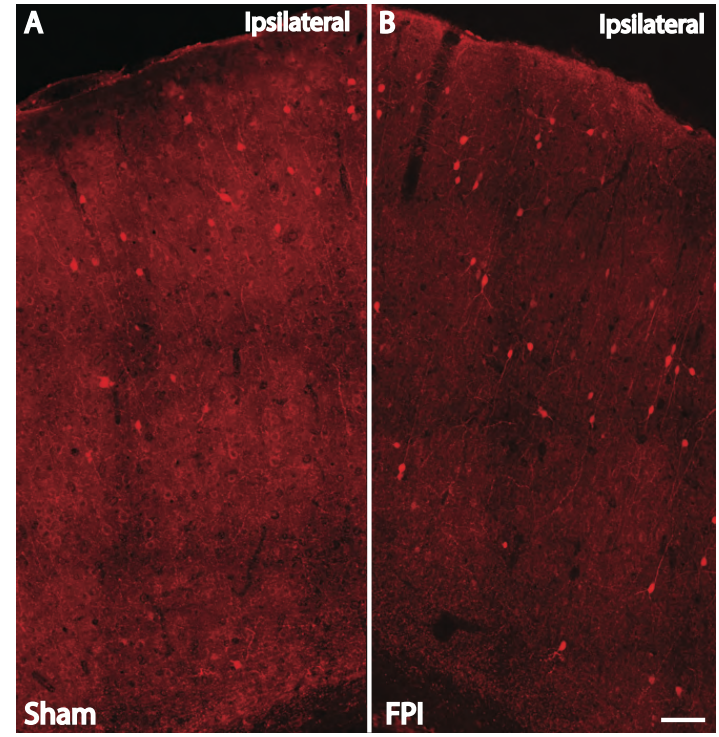
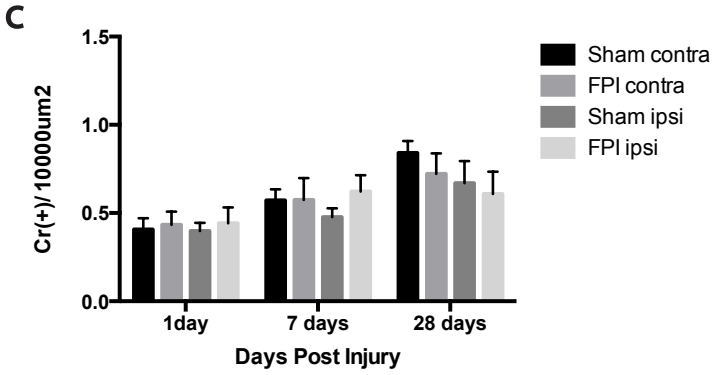
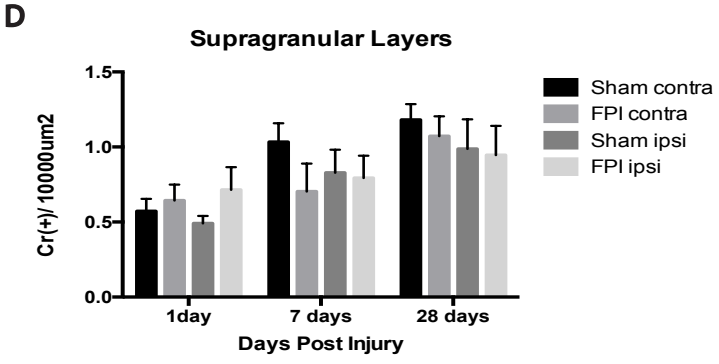

E
Infragranular Layers
GAD (+)/10000um2
0
2
4
6
1day
7 days
28 days
Days Post Injury
Sham contra
FPI contra
Sham ipsi
FPI ipsi

Figure 3.2. The number and distribution of interneurons is unaltered in the brain of GAD67-GFP mice.

Representative image of somatosensory cortex ipsilateral to the injury in sham (A) and FPI (B) mice at 7 days post-injury immunolabeled for GFP (A, B). The total number of GFP positive interneurons was determined in the cortex, in 3 sections ipsilateral and 3 sections contralateral to the injury site, from each of the 6 age-matched sham and 6 FPI mice that were analyzed per time point (C). Quantification of the density of interneurons in the superficial or supragranular layers of the cortex (layer I-IV) (D) and in the deep or infraganular layers of the cortex (layer V, VI) (E). Data are presented as mean ± SEM. Two-way ANOVA, followed by Bonferroni test. Scale bar=80μm.


A
Ipsilateral
B
Ipsilateral
Sham
FPI


C
Cr(+)/ 10000um2
1.5
1.0
0.5
0.0
1day
7 days
28 days
Days Post Injury
Sham contra
FPI contra
Sham ipsi
FPI ipsi


D
Supragranular Layers
Cr(+)/ 10000um2
1.5
1.0
0.5
0.0
1day
7 days
28 days
Days Post Injury
Sham contra
FPI contra
Sham ipsi
FPI ipsi

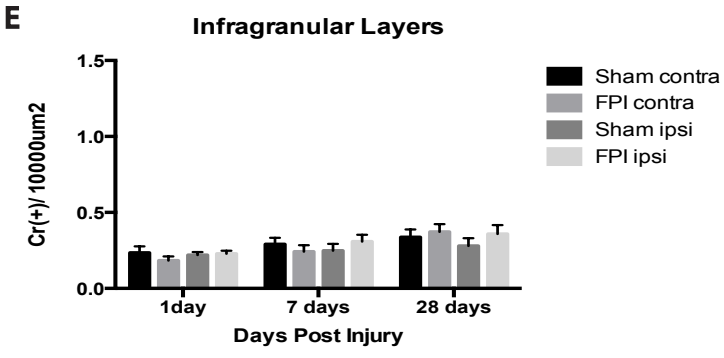

E
Infragranular Layers
Cr(+)/ 10000um2
1.5
1.0
0.5
0.0
1day
7 days
28 days
Days Post Injury
Sham contra
FPI contra
Sham ipsi
FPI ipsi

Figure 3.3. Mild brain injury does not alter Calretinin interneuron number in any layer of the somatosensory cortex of GAD67-GFP mice.

Representative image of ipsilateral cortical tissue derived from sham and FPI mice at 7 days post-injury immunolabeled for Calretinin (A, B). The total number of Calretinin interneurons was determined across all layers of the cortex in 3 sections ipsilateral and 3 sections contralateral to the injury site from each of the 6 age-matched sham and 6 FPI mice that were analyzed per time point (C). The number of Calretinin interneurons per area of cortex was also determined in the superficial or supragranuar layers of the cortex (layer I-IV) (D) and in the deep or infraganular layers of the cortex (layer V, VI) (E). Data are presented as mean ± SEM. Two-way ANOVA, followed by Bonferroni test. Scale bar=80μm.

A more detailed analysis of the number of dendritic branches and length of individual dendritic branches of Calretinin interneurons was carried out in order to determine if the differences found in total dendritic length in the injured cortex were due to a reduction in individual branch length or a possible decrease in cell complexity reflected in reduced branch number. Results indicate that Calretinin interneurons in the injured cortex at 7 days post-injury had a reduced dendritic complexity given that a reduction of secondary order dendrites in the ipsilateral side of the FPI mice was found (Fig 3.5 D, 2-way ANOVA, $p<0.02$). No differences were found in dendritic arbor complexity at 28 days post-injury (Fig 3.5 E, Two-way ANOVA, $p=0.09$).

Comparisons of Calretinin interneurons dendritic branch number at different time points post-injury indicate that the changes in dendritic arbor complexity present at 7 days post-injury were no longer detected at 28 days post-injury. Calretinin interneurons returned to control levels of complexity by 28 days post-injury. There was a significant increase in the number of secondary order processes at 28 days post-injury compared to 7 days post-injury ipsilateral and contralateral to the injury site and an increase in tertiary order processes ipsilateral to the injury site at 28 days post-injury respect to 7 days post-injury (Fig 3.5 F, G Two-way ANOVA, $p<0.004$). Moreover there were no differences found in soma size or length of individual dendritic branches in the injured cortex compared to sham controls at any of the post-injury time points analyzed (Fig 3.5 F, G Two-way ANOVA, $p=0.49$, $p=0.58$, respectively).

Overall it was determined that Calretinin interneurons in the injured cortex at 7 days post-injury were significantly less complex (Fig 3.5 J) than Calretinin interneurons in the sham control mice (Fig 3.5 K). By 28 days post-injury Calretinin interneurons were more complex than at 7 days post-injury and had regained a level of complexity similar to Calretinin neurons analyzed in the sham control animals.

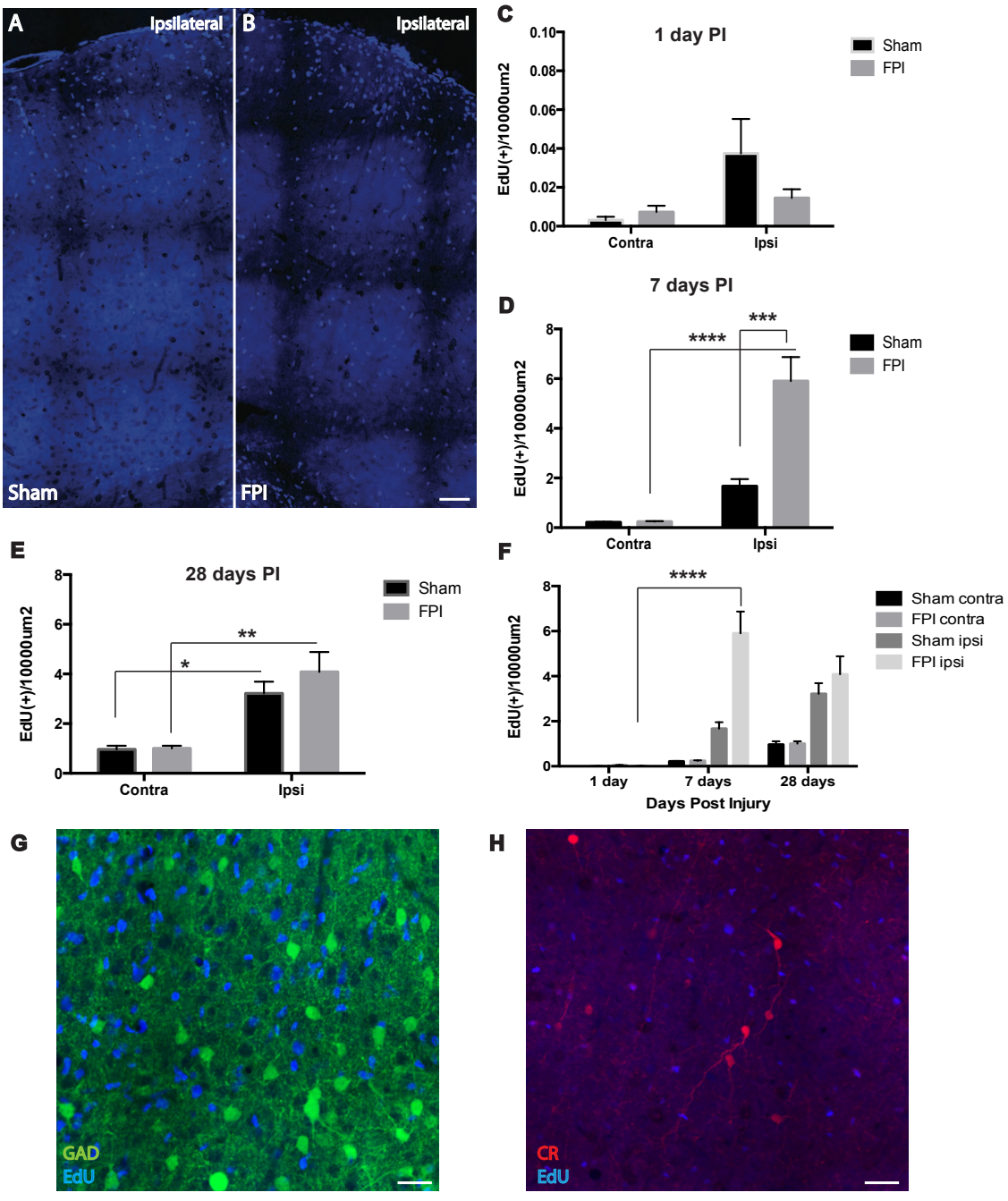


Figure 3.4. Mild TBI does not stimulate cortical interneuron generation in the mature brain.

In order to visualize cells born following injury, GAD67-GFP mice received EdU via the drinking water for 1, 7 or 28 days post-injury. Representative image of EdU$^+$ cells in cortical tissue derived from sham and FPI mice after 7 days of labeling (A, B). Scale bar=80μm. Quantification of the number of EdU$^+$ cells in the somatosensory cortex of sham or FPI mice revealed no significant increase in the number of EdU

*labeled cells at 1day post-injury (C). There was a significant increase in the number of EdU labeled cells at 7 days post-injury in the ipsilateral cortex of FPI mice compared to the contralateral side and the ipsilateral cortex of sham mice (D). At 28 days post-injury there was a significant increase in the number of EdU^+ cells in the ipsilateral cortex of sham and FPI mice compared to the contralateral cortex (E). Analysis of EdU^+ cells over time showed a significant increase at 7 days post-injury in the ipsilateral cortex of FPI mice relative to 1 day post-injury and no overall significant changes at 28 days post-injury compared to 7 days post-injury (F). Representative images of the ipsilateral cortex of GAD67-GFP mice after mild FPI immunolabeled for GFP^+ EdU^+ and CR^+ EdU^+ (G, H). Two-way ANOVA, followed by Bonferroni test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM Scale bar=60µm.*

3.3.6. Reduced synaptic inhibition in layer V pyramidal neurons after mild TBI

To determine whether the alterations in the dendritic arbor of Calretinin interneurons after injury reflect a decrease in synaptic transmission in the injury site, whole cell patch clamp recordings from Layer V pyramidal neurons from sham and FPI *Thy1-YFP-H or GAD67-GFP* mice were made. FPI animals received a pulse of pressure onto the dura of 1.46 ± 0.04 atmospheres. There was a significant difference between the righting time of sham (0.13 ± 0.02 minutes) and FPI animals (3.06 ± 1.12 minutes; $p<0.0001$) indicating that FPI mice had received a mild cortical injury (Alder et al., 2011). There were no significant differences in age or weight between the sham (age 10.4 ± 0.31 weeks; weight 27 ± 0.71 grams, (n=5)) and FPI groups (age 10.3 ± 0.13 weeks; weight 28.8 ± 0.34 grams, (n=7)) exposed to mild brain injury. During electrophysiological recordings neurons were filled with Neurobiotin allowing for post-recording histological characterization of the cell to confirm its location in layer V of the somatosensory cortex in the region underlying the craniectomy (Fig 3.6 A, B).

Electrophysiological data revealed no significant differences in capacitance (sham 58.5pF ± 9.2pF, FPI 48.1pF ± 3.5pF, $p=0.268$; two-tailed t-test (data not shown)) in the injured group compared to the sham group. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded from Layer V pyramidal neurons from sham and FPI mice at 7 days post-injury (Fig 3.6 C). The average amplitude of the mIPSCs was equivalent between sham (12.7pA ± 0.8pA) and injured mice (13.5pA ± 0.7pA) ($p=0.491$, two-tailed t-test, Fig 3.6 D). Pyramidal neurons in the somatosensory cortex of injured mice received significantly fewer inhibitory synaptic inputs compared to sham mice as evidenced by a decrease in frequency of mIPSCs from 1.46 ± 0.26 Hz to 0.68 ± 0.12 Hz ($p<0.0061$, two tailed t-test, Fig 3.6 E). There were no differences found in mIPSCs rise (sham 4.33ms ± 0.64ms, FPI 4.78ms ± 0.71ms, $p=0.67$, two tailed t-test, Fig 3.6 F) or decay time (sham 7.38ms ± 0.95ms, FPI 13.32ms ± 2.74ms, $p=0.14$, Paired t-test, Fig 3.6 G).

Overall results showed alterations in mIPSCs frequency but not amplitude after mild brain injury.

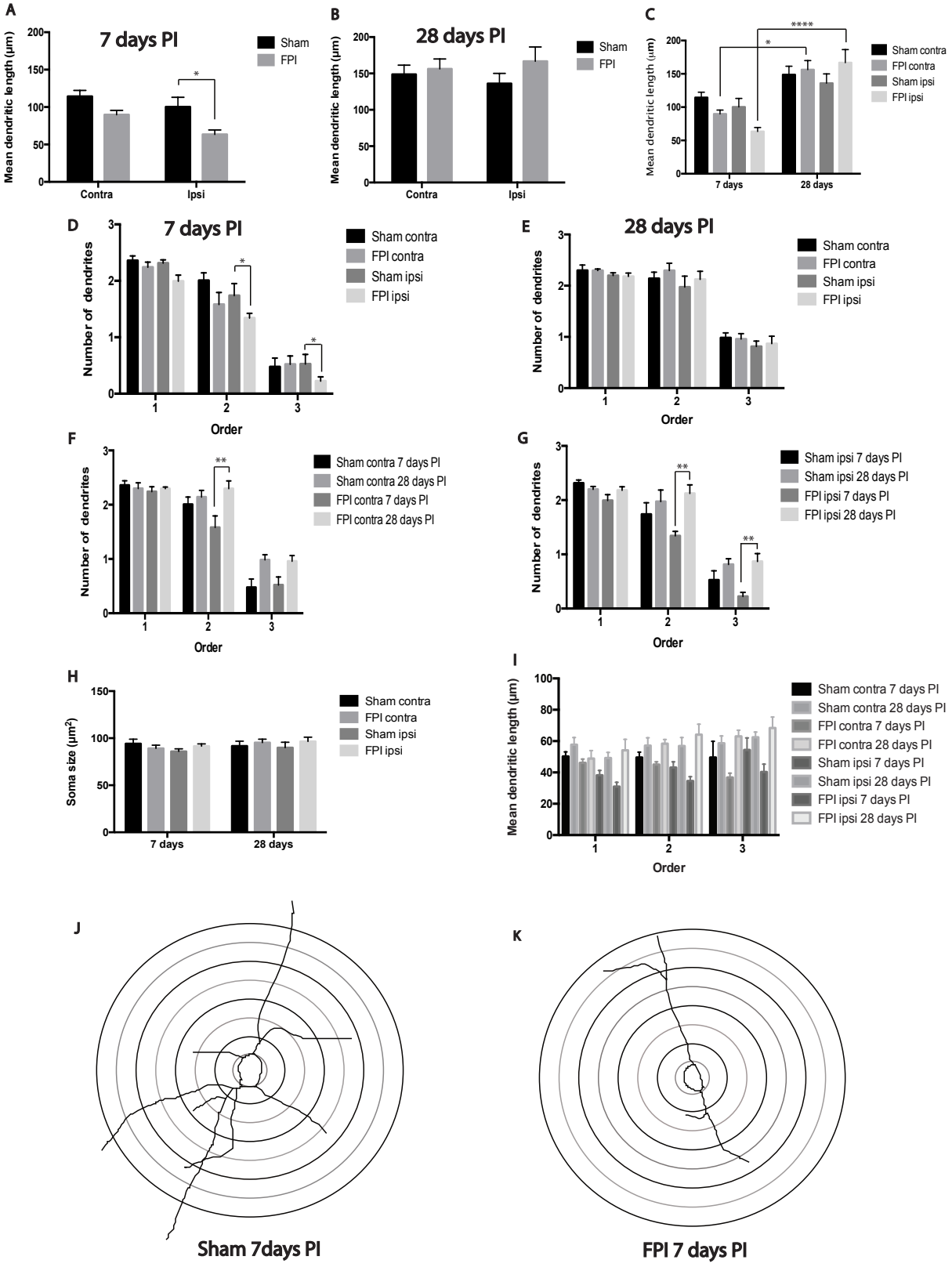

A
7 days PI
Mean dendritic length (µm)
Sham
FPI
Contra
Ipsi
B
28 days PI
C
Sham contra
FPI contra
Sham ipsi
FPI ipsi
7 days
28 days
D
Number of dendrites
Order
E
F
Sham contra 7 days PI
Sham contra 28 days PI
FPI contra 7 days PI
FPI contra 28 days PI
G
Sham ipsi 7 days PI
Sham ipsi 28 days PI
FPI ipsi 7 days PI
FPI ipsi 28 days PI
H
Soma size (µm²)
I
J
Sham 7days PI
K
FPI 7 days PI

Figure 3.5. Calretinin interneuron dendritic arbor complexity analysis at 7 and 28 days after mild TBI.

*Calretinin interneurons were traced using Neurolucida software in order to investigate their dendritic morphology at 7 and 28 days post-injury. There was a significant decrease in total dendritic length in Calretinin interneurons located in the ipsilateral side in FPI mice compared to sham mice at 7 days post-injury (A, p<0.05). There were no differences in mean dendritic length found at 28 days post-injury between the same groups (B). Over time analysis of total dendritic length at 7 and 28 days post-injury of FPI cortex contralateral and ipsilateral to the injury site at 7 days post-injury compared to cells found in the same regions at 28 days post-injury (C). Number of primary, secondary and tertiary dendrites of Calretinin interneurons at 7 (D) and 28 days post-injury (E). Over time analysis of dendritic arbor complexity of Calretinin interneurons in the contralateral cortex (F) and the ipsilateral cortex (G) of sham mice and FPI mice. Soma size analysis of Calretinin interneurons in the cortex of sham and FPI mice at 7 or 28 days post-injury (H). Length analysis of primary, secondary or tertiary order processes of Calretinin interneurons at 7 or 28 days post-injury in sham and FPI mice (I). Graphical representation of Calretinin interneurons in the ipsilateral cortex of sham and FPI mice at 7 days post-injury (J, K). Each concentric circle represents 10μm from the cell soma. Data are presented as mean ± SEM. Two-way ANOVA, followed by Bonferroni test *p<0.05, **p<0.01, ****p<0.0001.*

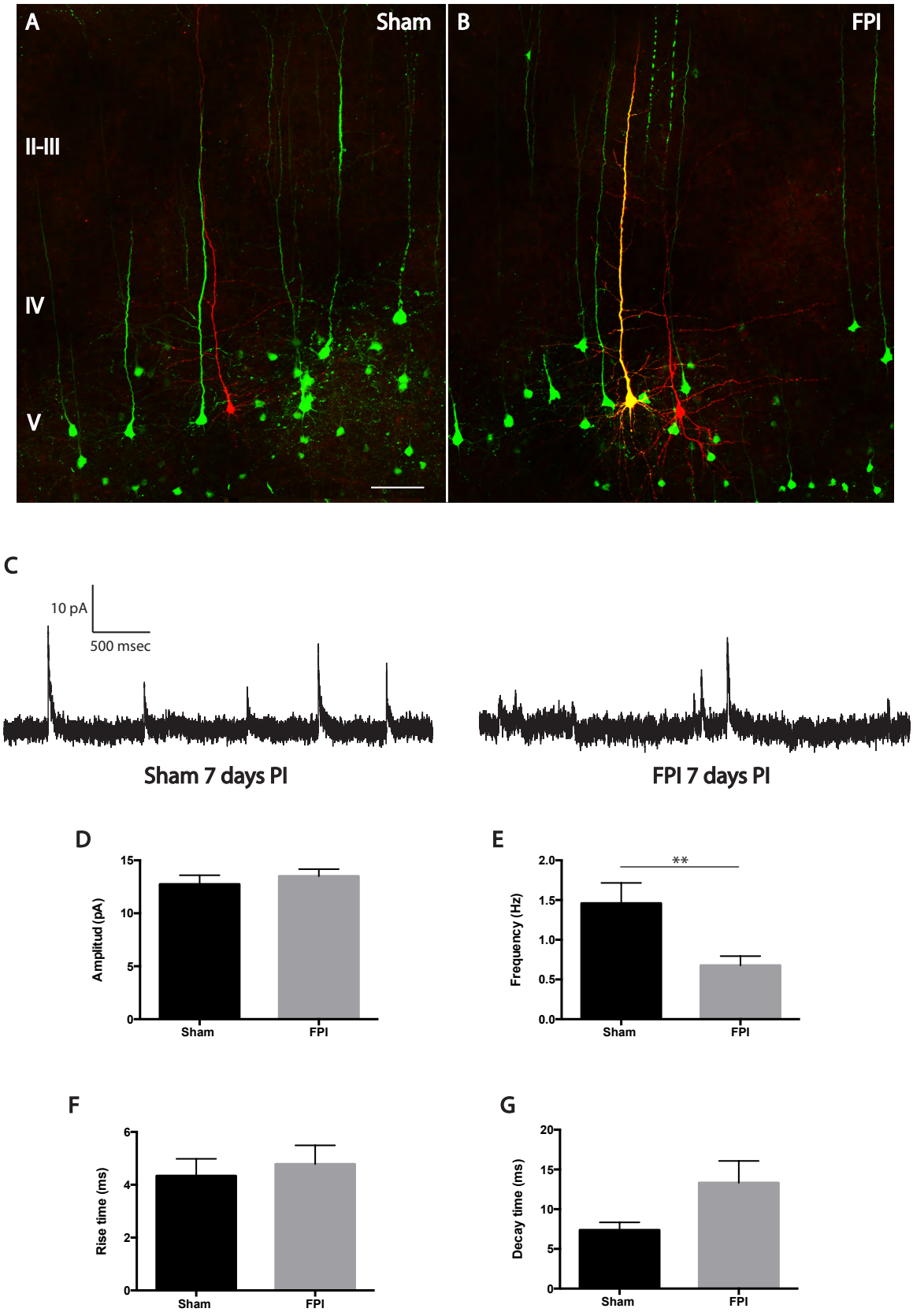

A
Sham
II-III
IV
V
B
FPI
C
10 pA
500 msec
Sham 7 days PI
FPI 7 days PI
D
Amplitud (pA)
15
10
5
0
Sham
FPI
E
Frequency (Hz)
2.0
1.5
1.0
0.5
0.0
**
Sham
FPI
F
Rise time (ms)
6
4
2
0
Sham
FPI
G
Decay time (ms)
20
15
10
5
0
Sham
FPI

Fig 3.6. Mild TBI results in a loss of inhibitory input to layer V pyramidal neurons

*Whole cell patch clamp recordings from layer V pyramidal neurons from sham and FPI mice were taken. During recordings neurons were filled with Neurobiotin and post stained to confirm their location in layer V and in the injury site. Representative image of layer V filled neurons after whole cell patch clamping (A, B). Example traces from sham and FPI mice showing mIPSCs (C). mIPSCs amplitude (Sham 12.74 ± 0.68pA, FPI 13.50 ± 0.67pA) (D), frequency (Sham (n=8 cells from 5 animals) 1.46 ± 0.25 Hz, FPI (n=12 cells from 8 animals) 0.68 ± 0.12 Hz) (E), and rise time (Sham 4.34 ± 0.64ms, FPI 4.78 ± 0.71ms) (F), decay time (Sham 7.38 ± 0.95ms, FPI 13.32 ± 2.74ms) (G) . Data are presented as mean ± SEM. Paired t-test **p<0.01. Scale bar= 100µm.*

3.4. DISCUSSION

Mild TBI can lead to cognitive and neuropsychiatric impairments in humans despite the absence of clear structural damage (Li et al., 2016). In this Chapter the investigations highlight that after a single mild brain injury a distinct mechanism of injury-induced neuronal plasticity in a discrete subpopulation of cortical interneurons is activated and is linked to decreased inhibitory input in pyramidal neurons.

In this Chapter a Fluid Percussion Injury (FPI) device was used to induce a mild lateral brain injury in *GAD67-GFP* and *Thy1-YFPH* mice and investigate post-injury alterations in interneurons. Mild injuries to the brain were confirmed by the lack of clear structural damage, absence of post-injury mortality and the short time required for mice to regain their righting reflex post-TBI (Thompson et al., 2005, Alder et al., 2011) (Table 3.1, Fig 3.1) . Lateral FPI is a well-characterized model of brain injury that reproduces key features of human TBI including focal contusion, subarachnoid hemorrhages, gliosis, neuronal loss and axonal injury (Dixon et al., 1987, Hayes et al., 1987, Pitkanen and Immonen, 2014).

Several studies have reported a deficit in GABAergic activity and loss of GABAergic interneurons in the hippocampus following various types of injury (Witgen et al., 2005, Mtchedlishvili et al., 2010, Pavlov et al., 2011, Almeida-Suhett et al., 2015). There is also evidence that severe injuries induce changes in the total number of interneurons and in specific populations of interneurons in the cortex (Cantu et al., 2015). However, alterations in the cortical inhibitory circuit after mild brain injury have not been previously evaluated. Therefore, in this Chapter, investigations aimed to quantify alterations in the population of interneurons after mild TBI. Results indicate that mild brain injury does not induce changes in the total number of interneurons or specific subpopulations of interneurons that reside in different layers of the cortex shortly after injury or as a delayed mechanism at later post-injury time points (Fig 3.2). Moreover, there was no change in the number of Calretinin interneurons at any of the post-injury time points analyzed (Fig 3.3). Hence it was concluded that mild injury to the brain did not induce overall changes in the number of cortical GABAergic interneurons.

Lateral FPI produces both focal and diffuse effects. The brain regions ipsilateral or under the injury site undergo most of the damage, while more distant parts of the brain, like the contralateral hemisphere, are usually not overtly affected (Peterson et al., 2015, Ostergard et al., 2016). It has previously been reported that

severe FPI can induce important reactive astrogliosis in the cortex characterized by low baseline levels in sham animals and the contralateral hemisphere and modest increases in trauma animals, peaking at 7 days and decreasing by 30 days post-injury (Carbonell and Grady, 1999). In this Chapter cell proliferation in the cortex was evaluated as the presence of newly generated EdU^+ cells and based on the literature, was used as an indication of a post-injury glial response (Chirumamilla et al., 2004, Rice et al., 2003, Susarla et al., 2014). It was determined that mild FPI induces cell proliferation under the injury site but not in other regions of the cortex, including the contralateral hemisphere. These results suggest more focal than diffuse damage after mild lateral FPI (Fig 3.4).

Several studies have observed neurogenesis in rodents in response to moderate to severe traumatic brain injury (Dash et al., 2001, Rice et al., 2003, Blaiss et al., 2011, Cope et al., 2016, Wang et al., 2016). However, most studies have indicated that neurogenesis does not lead to neuronal replacement in the cortex (Salman et al., 2004, Blizzard et al., 2011). Since the focus of the current study is the inhibitory network the presence of newly generated cortical interneurons was evaluated. There was no induction of interneuron neurogenesis after injury indicated by the lack of EdU^+ GFP^+ and EdU^+ CR^+ neurons in the cortex. It was therefore concluded that despite notable cell proliferation, mild TBI evoked by lateral FPI in the mouse brain does not induce neurogenesis in the cortex (Fig 3.4).

Previous studies have indicated that cortical GABAergic interneurons have the capacity to remodel their dendritic arbors under physiologically normal and pathological conditions (Lee et al., 2006, Lee et al., 2008, Blizzard et al., 2011, Chen et al., 2011). Moreover dendritic remodeling in cortical interneurons that express Calretinin was demonstrated in Chapter 2 using an *in vitro* model of transection injury. In this Chapter results indicate that mild FPI also induces morphological changes in Calretinin interneurons. Results showed that at 7 days post-injury Calretinin interneurons in the injury site had fewer secondary and tertiary processes when compared to the contralateral cortex and sham animals. However, those changes in dendritic arbor complexity were time dependent and were restored to sham levels by 28 days post-injury (Fig 3.5). During early post-natal development glutamate-mediated activity is essential for normal development of axonal and dendritic arbors in Calretinin interneurons (De Marco Garcia et al., 2011). Several studies have demonstrated an imbalance of excitation and inhibition shortly after injury (Ping and Jin, 2016) and increased glutamate levels (Hinzman et al., 2010,

Hinzman et al., 2012). It is plausible that after mild brain injury Calretinin interneurons revert to a developmental stage where changes in excitation can promote changes in dendritic arbor complexity.

Functional studies were carried out to determine if the morphological changes found in the subpopulation of Calretinin interneurons after mild brain injury could lead to functional alterations in the whole inhibitory circuit. For this purpose miniature inhibitory post-synaptic currents (IPSCs) were recorded from layer V pyramidal neurons. Recordings of the inhibitory activity or inhibitory afferents onto pyramidal neurons were used as an indication of the overall inhibitory network activity after injury (Cantu et al., 2014, Smith et al., 2015). Miniature IPSCs are spontaneously occurring events that reflect spontaneous, non action potential dependent release of GABA from individual presynaptic terminals of GABAergic interneurons (Cohen et al., 2007). The frequency, amplitude, rise time and decay time of mIPSCs were calculated. Results indicated that at 7 days post-injury the average amplitude of the mIPSCs was equivalent between sham and FPI mice. The equivalent mIPSCs amplitude found between groups suggests that the surface expression of GABA receptors in layer V pyramidal neurons after injury was likely unaffected. Mild TBI was associated with a significant decrease in mIPSCs frequency. The observed decrease in mIPSCs frequency indicates that the number of inhibitory connections onto layer V pyramidal neurons in the injured cortex was potentially decreased at 7 days post-injury (Fig 3.6).

Similar results have been reported for granule cells and pyramidal neurons in the hippocampus after both mild and severe brain injury, at similar post-injury time points (Witgen et al., 2005, Mtchedlishvili et al., 2010, Almeida-Suhett et al., 2015). So far few studies have focused on exploring cortical inhibitory synaptic transmission after mild brain injury. Smith et al. reported no changes in layer V pyramidal neuron mIPSC frequency and amplitude in the prefrontal cortex after mild brain injury. The apparent discrepancy between this report and results in this Chapter may lay in the fact that the prefrontal cortex was not within the injury site in that study (Smith et al., 2015). In the present investigation the alterations in inhibitory synaptic transmission were found in the injury site in the somatosensory cortex where the morphological changes in Calretinin interneurons dendritic arbors were observed. Moreover, the contrasting results might also reflect differences in neuroplasticity between the somatosensory cortex or barrel cortex and the prefrontal cortex (Erzurumlu, 2003, Medini, 2014, Sims et al., 2015).

Given that there were differences found between the sham and FPI groups in mIPSCs frequency but not mIPSCs amplitude, these results suggest that changes in inhibitory synapses onto layer V pyramidal neurons are predominantly presynaptic rather than postsynaptic, reflecting changes in the inhibitory network. A decrease in IPSCs frequency could be produced by either a decrease in the number of inhibitory synaptic afferents, or their efficacy onto the recorded cells, or a decrease in neurotransmitter release probability from these afferents, or a combination of both mechanisms. Although further experiments need to be carried out in order to determine the possible mechanism related to the decrease in mIPSCs frequency, previous studies in other regions of the brain, like the hippocampus, have determined that the IPSC pair pulse vesicle release is not affected after injury (Hunt et al 2011). Moreover there were no changes in mIPSCs kinetics (rise and decay time) indicating no changes in GABA receptor-subunit composition in the postsynaptic neuron further pointing to a presynaptic involvement (Hollrigel and Soltesz, 1997, Banerjee et al., 2013).

Calretinin interneurons represent approximately 10-30% of the total interneurons in the cortex (Markram et al., 2004). Even though this population of interneurons comprises a relatively small fraction of the total inhibitory neurons in the cortex, their unique connectivity and placement in the circuitry affords a large impact on inhibitory regulation as a whole (Dzaja et al., 2014). They have been described as being at the top of the interneuron hierarchy due to the fact that they preferentially regulate the activity of other interneuron populations (Barinka et al., 2015) and specifically target dendritic inhibitory interneurons to synchronize their activity (Caputi et al., 2009). This synchronization of dendritic inhibitory cells is a crucial process to provide effective inhibitory control of excitatory synaptic input of pyramidal cell dendrites (Toth and Magloczky, 2014). As a result Calretinin interneurons have a far-reaching and wide influence on the whole cortical inhibitory circuit (Toth et al., 2010). It is therefore hypothesized that the post-injury dendritic remodeling of Calretinin interneurons, in terms of loss of secondary and tertiary processes, could result in less effective synchronization of dendritic inhibitory interneuron firing, leading to less effective inhibition of pyramidal neuron firing and ultimately resulting in a pathologic shift toward excess excitability. The changes described in dendritic arbor complexity of Calretinin interneurons were transient and reverted by 28 days post-injury. Further studies would need to be carried out in order to determine if the changes in inhibitory synaptic transmission found at 7 days post-injury are still present at a later time point when compensatory mechanisms seem to be activated to restore normal dendritic

complexity and possibly compensate for changes in synaptic input and reestablish normal excitatory/inhibitory balance. Furthermore future studies would be aimed to investigate the possible behavioral and cognitive changes related to dendritic remodeling of Calretinin interneurons in mild TBI.

4. THE MICROTUBULE-STABILIZING DRUG EPOTHILONE D INCREASES AXONAL SPROUTING FOLLOWING TRANSECTION INJURY *IN VITRO*

4.1 INTRODUCTION

Axonal injury is a common consequence of traumatic brain injury (TBI). Axons are particularly vulnerable to injury as they project over long-distances and have a complex and highly organized structure (Meythaler et al., 2001, Johnson et al., 2012). After a traumatic brain injury the extent of damage is primarily determined by the severity of the initial injury. Although it is now recognized that even in the absence of initial axonal transection, and in seemingly mild forms of TBI, adverse axonal alterations occur at the level of the axon cytoskeleton (Smith et al., 2013). In particular, TBI can lead to cytoskeletal misalignment or loss, and the accumulation or compaction of cytoskeletal components which are hallmarks of neuronal degeneration (Maxwell, 1995).

Microtubule alterations after injury include the loss of microtubule-associated proteins (MAPs) such as Tau and MAP2, (King et al., 2000, Farkas and Povlishock, 2007, Bradke et al., 2012, Smith et al., 2013) and defects in axonal transport that lead to axonal swelling, the formation of axonal bulb structures, microtubule disconnection and ultimately axotomy (Smith et al., 1999, Tang-Schomer et al., 2010). There is evidence to suggest that following brain injury, changes in microtubule dynamics are a critical component in the cascade of events that lead to reactive gliosis and the formation of the glial scar (Chuckowree and Vickers, 2003, Blizzard et al., 2011). However, microtubule reorganization has also been implicated in attempted axonal regeneration following injury, specifically the elaboration of sprouts into and across the lesion site (Blizzard et al., 2011, Sengottuvel and Fischer, 2011).

The importance of microtubule deregulation in TBI pathology suggests that after injury, treatment with compounds that stabilize cytoskeletal microtubules can potentially modify multiple aspects of the brain's response to trauma. Microtubule stabilizing drugs, such as epothilones and taxanes, are widely used for cancer treatment and are now being investigated for their potential use in diseases of the nervous system. At high concentrations these drugs hyper-stabilize microtubules and interfere with their normal breakdown, which is critical for cell division (Goodin et al., 2004, Kolman, 2005). However, at low concentrations these same drugs can stabilize microtubules enough to prevent their depolarization and dissolution, and

even encourage their polymerization. This effect would be therapeutically significant for the treatment of the injured brain (Brunden et al., 2010, Hellal et al., 2011, Sengottuvel and Fischer, 2011, Baas and Ahmad, 2013).

Paclitaxel, one of the taxanes, has been used in various animal models of nervous system trauma, and has been shown to promote axonal elongation and regeneration, reduce glial scar formation, and improve outcomes after nerve injury (Hellal et al., 2011, Sengottuvel et al., 2011). Despite these encouraging findings, Paclitaxel is not an ideal drug for the treatment of TBI due to poor blood–brain barrier (BBB) permeability, which prevents access of the drug to the CNS. However Epothilone D (Epo D), a microtubule-stabilizing drug that competes with Paclitaxel for the binding site on β-tubulin, (Brunden et al., 2011) may be an excellent alternative. Epo D readily crosses the BBB and is retained within the CNS for several days (Wang et al., 2005b, Andrieux et al., 2006, Cortes and Baselga, 2007, Brunden et al., 2012). It has been administered to mice carrying genetic mutations associated with familial Alzheimer's disease and other related tauopathies, and was shown to compensate for the loss of tau function, improve axonal transport, reduce axonal dystrophy, decrease tau neuropathology, reduce neuronal loss and improve cognitive performance (Brunden et al., 2010, Barten et al., 2012, Lou et al., 2014). The beneficial effects of Epo D observed in these studies, coupled with the vital BBB penetration, suggest that this drug holds considerable promise as a potential therapeutic strategy for the treatment of TBI.

In this Chapter the effect of Epo D on the post-injury axonal sprouting response of cultured neurons was examined. Epo D was found to induce a regenerative response in neurons subjected to an *in vitro* model of CNS trauma. Results from this Chapter indicate that Epo D could find therapeutic efficacy in TBI, tackling the brain's inability to repair following injury by promoting the regeneration of neuronal sprouts, although further investigations using *in vivo* animal models of injury are necessary.

4.2. MATERIALS AND METHODS

4.2.1. Wild type and transgenic mice

All experimental procedures involving animals were approved by the Animal Ethics Committee for animal experimentation of the University of Tasmania and were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (A0011952). C57BL/6 wild type and *Thy1-YFP* transgenic mice were used for experiments. Animals were housed in individually ventilated cages, maintained at ~20^{o}C, on a 12 hour light/dark cycle, with access to food and water *ad libitum*.

4.2.2. Cell culture

Primary dissociated cortical neuron cultures were prepared from mouse embryos as previously described (Dickson et al., 2000). Briefly, C57BL/6 and *Thy1-YFP* transgenic mice were time-mated, the pregnant females killed by CO_2 exposure at 15.5 days of gestation, and the embryos removed. Embryos were genotyped by fluorescence imaging using 470nm light on a Carestream Image station 4000MM pro (Carestream Molecular Imaging, USA). Neocortical hemispheres were dissected into 5 ml Hanks Buffered Salt Solution (HBSS; Thermo-fisher Scientific, USA). Cortical tissue was dissociated by enzymatic digestion (0.025% w/v Trypsin, 5 minutes, 37^{0}C), which was halted by the addition of 1 ml of pre-warmed medium [Neurobasal^{TM} supplemented with 2% v/v B27, 10% v/v fetal calf serum, 0.5mM L-glutamine, 25µM glutamate and 1% streptomycin penicillin; Thermo-fisher Scientific, USA]. Tissue pieces were allowed to settle and after removal of the medium, dissociation was completed by resuspension of the cells in 500µl of pre-warmed medium and gentle trituration. Cell viability and concentration was assessed using trypan blue vital dye exclusion and 3.5 x 10^4 cells/mm^2 were plated per 19mm diameter glass coverslip (Marienfeld, Germany). Each coverslip was pre-coated with 0.001% Poly-L-lysine (Sigma-Aldrich, USA) in 0.01M PBS (pH 7.0).

Cultures were maintained in a 37°C humidified atmosphere of 5% CO_2 for up to 15 days. At 1 day *in vitro* (DIV) the medium was removed and replaced with serum-free growth medium [Neurobasal^{TM}, 2% v/v B-27 supplement, 0.5 mM L-glutamine, and 1% streptomycin penicillin; Thermo-fisher Scientific, USA], after which time ~50% of the medium was replaced every 3–4 days.

4.2.3. Axonal transection and pharmacological treatment of cortical neurons

Axonal transection was carried out using a Barkan goniotomy knife (Kaisers, Germany) on cortical cultures at 15 DIV (Blizzard et al., 2013). One long injury that extended the full diameter of the coverslip was made, ensuring complete axonal transection and producing a cell free lesion of ~50-150μm wide. Primary cultures were immediately treated with 0.1nM, 1nM, 10nM or 100nM Epo D or dimethylsulphoxide (DMSO; Sigma) alone, which acted as a vehicle control. Twenty-four hours after treatment, cells were fixed for 30 minutes with 4% (w/v) (PFA) in (PBS). Each treatment was performed on four replicate coverslips. Analysis was performed in four replicates across three independent experiments.

4.2.4. Cell viability assays

The Alamar blue (Invitrogen) assay, an indicator of cellular metabolic activity, was used to measure the viability of cultured neurons after drug treatment. Cells were grown at a density of 2 $x10^5$ cells/mm^2 in 96 well plates. At 15 DIV they were treated with increasing concentrations of Epo D. 24 hours after treatment cells were washed three times with Neurobasal™ medium and incubated for 4 hours at 37°C with a 10% (v/v) dilution of Alamar blue in serum-free growth medium. Fluorescence was detected using a Fluostar optima automated plate-reading fluorometer (BMG labtech), set to 540nm excitation and 590nm emission detection, and analysis was performed in triplicates on three independent experiments.

Quantification of pyknotic nuclei was performed on cultured neurons fixed with 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS). Neurons were stained with the fluorescent DNA-binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen, Life technologies, USA) at 1:1000 dilution in PBS for 15 minutes at room temperature. Neurons with condensed or fragmented nuclei were considered pyknotic. Two hundred cells were counted per culture, and a minimum of four independent cultures analyzed per treatment condition.

4.2.5. Western blot analysis

Cortical neurons were grown on 12 well plates at a density of 5 x 10^5 cells/mm^2. At 15 DIV neurons were treated with 0.1nM Epo D, 100nM of Epo D, or DMSO for 90 minutes or 24 hours. Cultures were subsequently washed 3 times in warmed serum-free medium and placed on ice. The serum-free medium was removed and 150 μL of cell lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 16 mM nonyl phenoxypolyethoxylethanol-40 (NP-40), 24 mM Sodium deoxycholate, 3.5 mM

SDS, and one protease inhibitor tablet (Roche Diagnostics Pty Ltd, Australia) containing Trichostatin A (TSA; 1:10000, Sigma)] was added to each well. Cells from each well were scraped, transferred to a 1.5ml microcentrifuge tube and centrifuged at 13,000 RPM 4°C for 10 minutes (Hermle Z160M, Hermle Labortechnick, Germany). The supernatant was removed and the protein content quantified using a Bio-Rad-DC assay in microplate format (Bio-Rad Pty Ltd, Australia).

Samples were diluted in Laemelli sample buffer (Bio-Rad, New Zealand), vortexed, and heated to 90°C for 10 minutes. The samples were next separated on a 12% SDS-PAGE gel by sodium dodecyl sodium sulfate polyacrylamide gel electrophoresis at 100V for 60 minutes and 150V for 20 minutes. Proteins were electrophoretically transferred to nitrocellulose membranes at 80V for 60 minutes at 4°C and blotted with antibodies against Acetylated-tubulin (1:5000; Sigma-Aldrich), Tau (1:5000; Dako), and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase; 1:10000, Merck Millipore) diluted in TBST (TBS, 0.05% v/v Tween- 20) containing 5% w/v skim milk powder and 0.05% v/v Tween-20, overnight at 4°C. After washing three times for 10 minutes in TBST, membranes were incubated with peroxidase-conjugated secondary antibodies diluted in TBST (1:5000, Dako, anti-rabbit HRP, anti-mouse HRP) for 90 minutes at room temperature. The chemiluminescent signal was developed using the Immobilon Western Chemiluminescent HRP Substrate (Merk Millipore) according to the manufacturer's instructions, and detected using a Chemi-Smart 5000 instrument (Vilber Lourmat, France). Images were analyzed using ImageJ 1.46. Experiments were performed in triplicates.

4.2.6. Immunocytochemistry

Cultures at 15 DIV that were either untreated, or injured and exposed to Epo D or DMSO for 24 hours were fixed in 4% w/v PFA in PBS prior to immunolabeling. Primary antibodies used included rabbit anti-Tau (1:10,000; Dako); rat anti-green fluorescent protein to label YFP (1:3,000; Nacalai tesque); and rabbit anti-synaptophysin (1:1000; Millipore), diluted in 0.3% v/v Triton-X100 (Fluka) in PBS, and applied for 1 hour at room temperature followed by overnight incubation at 4°C. Coverslips were washed thrice in PBS before the application of isotype- and species-specific secondary antibodies. Secondary antibodies [Alexa Fluor® 594- and AlexaFluor® 488- conjugated goat anti-rat and goat anti-rabbit IgG H+L (Invitrogen)] were diluted 1:1000 in 0.01M PBS and applied to coverslips for 2 hours at room temperature. Coverslips were washed thrice with PBS before mounting in fluorescent mounting medium (Thermo scientific, USA). Immunolabeling was

visualized and imaged using a DMLB2 upright fluorescent microscope (Leica, Germany) or UltraView Spinning disk confocal microscope with Velocity Software (Perkin Elmer, USA).

4.2.7. Live cell imaging

Cells were grown in custom-made poly dimethylsiloxane (PDMS) chambers placed over 22 mm x 22 mm poly-L-lysine coated glass coverslips, and plated at a density of $1.5x10^5$ cells/mm^2. Axonal transection was carried out using a Barkan goniotomy knife and treated with Epo D or DMSO (as described above). Cultures were imaged at 37°C, at a rate of 1 image every 10 minutes for a period of 24 hours using a Nikon TiE motorized inverted microscope with x40 / 0.95 objective. Each PDMS chamber contained 3 to 4 openings allowing injured cultures that were drug treated, and those that were vehicle treated, to be imaged during the same imaging session. Neurite growth rate and length was calculated using DIC images of post-injury neurites growing into the injury site, and Nikon NIS-elements AR 3.2 software (Nikon Instruments INC, USA).

4.2.8. Statistical analysis

All statistical analysis was performed in Graph-pad Prism version 6.04 (Graph-pad Software Inc., CA). Comparisons were made using a one-way ANOVA or Two-way ANOVA with Bonferroni post-hoc test or Student's t-tests as specified.

4.3. RESULTS

4.3.1. Cortical neuron development *in vitro*

Cortical neurons derived from *Thy1-YFP* transgenic mice were grown on poly-L-lysine coated coverslips, and in the days after plating, developed and extended neurites as previously described (Dotti et al., 1988). The morphological and histological characteristics of the neurons at 7 and 15 DIV were analyzed to characterize maturation. By 7 DIV neurons had extended long processes and had made contact with several other cells in the culture (Fig 4.1). However, by immunocytochemistry it was determined that the expression of the vesicular synaptic marker synaptophysin was minimal and diffuse (non-punctate) and found in the cell bodies and neurites (Fig 4.1 A-C). This expression pattern is indicative of neurons that are immature and have not formed synapses. In contrast, neurons cultured for 15 DIV were not only more complex, with multiple processes contacting surrounding cells, but the synaptophysin staining had become punctate and extended along the dendritic shafts (Fig 4.1 D-F). This pattern of staining is consistent with the synaptic expression of synaptophysin (Thiel, 1993).

4.3.2. Cell viability after drug treatment

To determine the concentrations of Epo D that were safe and efficacious the effect of Epo D addition on the viability of cultured cortical neurons was examined. The effect of Epo D on neuron viability was assessed using two different methods. The first examined the number of pyknotic nuclei in the culture, identified by nuclear morphology following DAPI staining (Fig 4.2 A-D). The percentage of neurons with pyknotic nuclei was equivalent between vehicle- and Epo D-treated cultures, being ~35% in vehicle-treated cultures and ranging from ~32% to ~40% for cultures exposed to a range of Epo D concentrations (Fig 4.2 E).

The second viability assay employed, utilized the fluorogenic redox indicator dye, Alamar blue. This dye becomes fluorescent upon reduction by mitochondrial enzymes and is an indicator of the metabolic activity of viable cells (Rampersad, 2012). The fluorescence intensity of vehicle- and Epo D-treated cultures was assessed and found to be equivalent (Fig. 4.2 F). Therefore neuron viability was unaffected by Epo D exposure at any of the concentrations trialed.

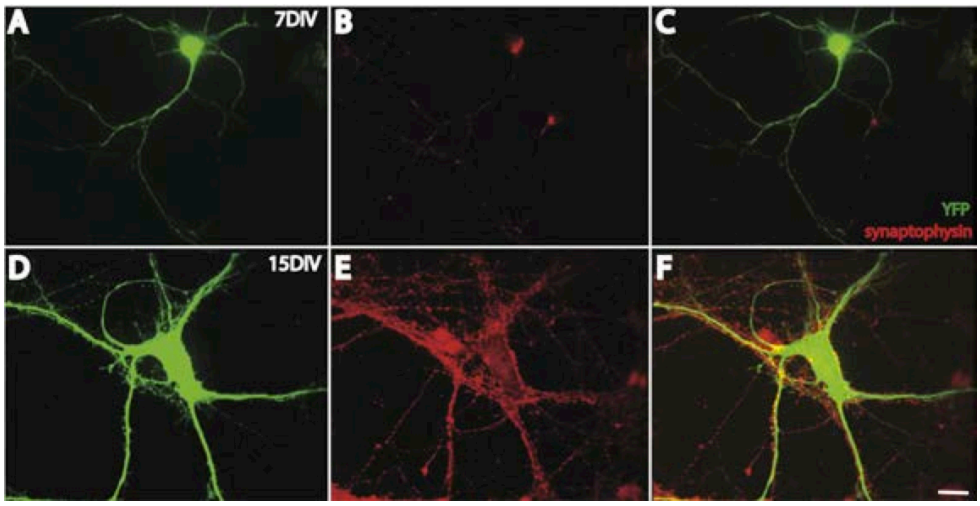


Figure 4.1. Development of Thy1-YFP cortical neurons in vitro.

To examine neuronal characteristics, dissociated YFP positive neurons were grown as a monolayer on a poly-L-lysine substrate. 7 DIV (A), 15 DIV (D) YFP labeling (green). 7 DIV (B), 15 DIV (E) labeling for the synaptic vesicle marker synaptophysin (red). 7 DIV(C), 15 DIV (F) double labeling for YFP and synaptophysin. At 7DIV double labeling for YFP and synaptophysin indicated that YFP was present throughout cell bodies and processes while there was minimal immunoreactivity for synaptophysin (C). At 15 DIV there was an increased immunoreactivity for synaptophysin (red) and there was a colocalization of synaptophysin and YFP in punctate positions lateral to dendrite shafts (F). Scale bar = 14 µm.

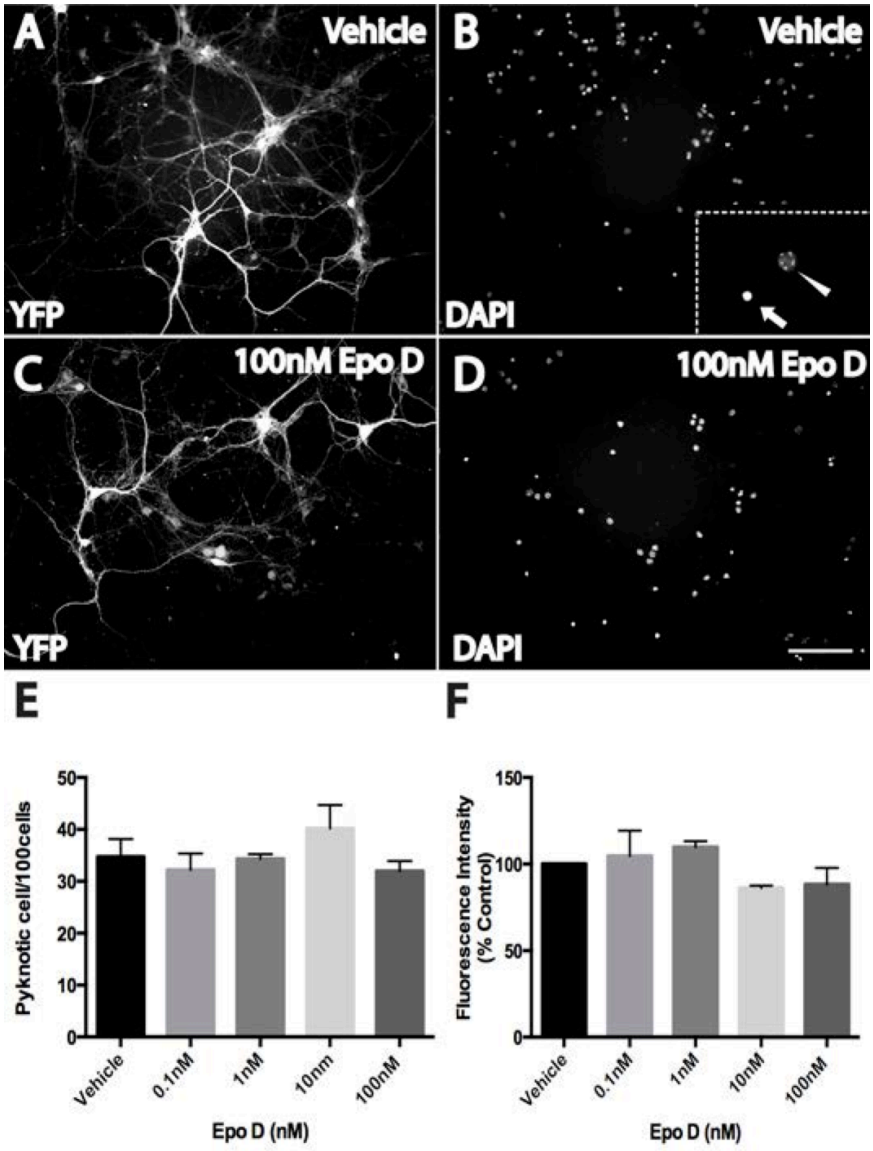


Figure 4.2. Cell viability after Epo D treatment.

Representative images of YFP positive neurons in culture at 15 DIV after vehicle treatment (A) or 100nM Epo D treatment (C). DAPI staining of 15 DIV neurons after vehicle treatment (B) or 100nM Epo D treatment (D). DAPI staining was used to evaluate the number of pyknotic cells; cells brightly stained with condensed and fragmented nuclei were considered pyknotic (Inset: apoptotic nuclei, arrow and normal nuclei, arrowhead). Bar graphs demonstrating the effect on cell viability of exposing cultures to Epo D for 24 hours. DAPI staining, evaluation of the percentage of pyknotic cells after drug treatment (E). Alamar blue fluorescence intensity was also used to assess neuronal viability (F). Fluorescence intensity was expressed as percentage of the control treatment. One-Way ANOVA followed by Bonferroni test. Data are presented as mean ± SEM . Scale bar=50μm.

4.3.3. Evaluation of Epo D effects on microtubule stabilization

To determine the efficacy of Epo D on microtubule (MT) stabilization in uninjured cortical neurons *in vitro*, the effect of Epo D treatment on the expression of a biomarker of MT stabilization, tubulin acetylation was investigated (Brunden et al., 2011). After 90 minutes of exposure to 0.1nM Epo D, cultured neurons were found to express the same level of acetylated tubulin as vehicle-treated control cultures (Fig 4.3 A). However treatment with 100nM Epo D resulted in a significant increase in the level of acetylated tubulin when compared to vehicle ($p=0.039$, ANOVA, n=3 cultures), which is suggestive of increased microtubule stabilization with high concentrations of the drug. There was no significant increase in acetylated tubulin in response to either concentration of Epo D when cultures were treated for 24 hours (Fig 4.3 B).

In order to determine whether the transient change observed in acetylated tubulin expression following Epo D treatment correlated with a change in Tau-protein expression, and to gain insight into the mechanism of action of the drug the influence that Epo D addition to cortical neurons exerted on total Tau protein levels was examined. After 90 minutes of treatment there was no change in the level of Tau protein expressed by cortical neuron cultures, when comparing Epo D- with vehicle-treated control neurons (Fig 4.3 C). However following 24 hours of exposure to 100nM Epo D, there was a significant decrease in total Tau protein levels (Fig 4.3 D; $p<0.01$, ANOVA, n=3). Therefore it was concluded that cortical neurons respond to 100nM Epo D treatment in the short-term by transiently increasing their expression of acetylated tubulin, which is indicative of stabilized microtubules. However sustained exposure to the drug for 24 hours reduced Tau protein levels.

4.3.4. Epo D modulates post-injury sprout outgrowth

Having demonstrated that Epo D can modulate tubulin stabilization, the effect of this microtubule stabilization on post-injury axonal sprouting response was investigated next. Cultures were grown for 15 DIV, to reach a state of relative maturity, before they were subjected to axonal transection. Epo D or vehicle was immediately added to the injured cultures, which were fixed 24 hours later for immunocytochemistry. Previous studies have indicated that 24 hours post-injury is the optimal time to evaluate sprouting, as this is when it is maximal (Chuckowree and Vickers, 2003, Blizzard et al., 2013). Tau immunolabeling revealed that Epo D exposure had a substantial effect on post-injury axonal sprouting.

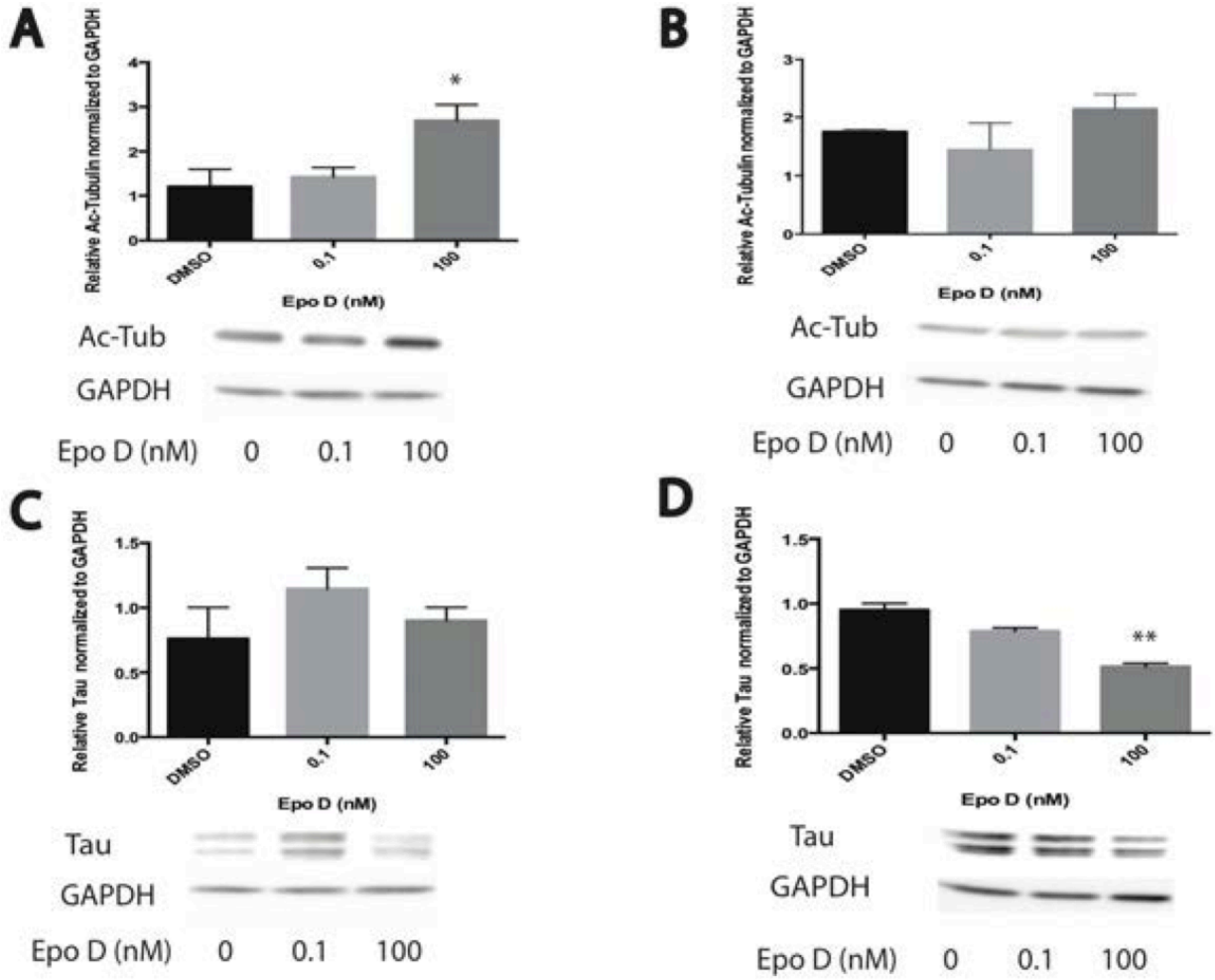


Figure 4.3. Evaluation of Epo D effects on acetylated tubulin and Tau levels.

*15 DIV cortical neurons were treated for 90 minutes and 24 hours with two doses of Epo D (0.1nM-100nM). Immunoblot and densitometry analysis of acetylated tubulin (Ac-Tub) (55kDa) and loading control GADPH (37kDa) 90 minutes post Epo D treatment (A) and 24 hours post drug treatment (B). Immunoblot and densitometry analysis of Tau (45-65 kDa) and GADPH at 90 minutes post Epo D treatment (C) and 24 hours post Epo D treatment (D). Data are presented as mean ± SEM . One way ANOVA, followed by Bonferroni test *p<0.05, **p<0.01.*

In vehicle-treated cultures there was axonal sprout outgrowth into the injury site 24 hours after injury (Fig 4.4 A), and this appeared to be modulated by Epo D-treatment (Fig 4.4 B-D). The number of sprouts identified and quantified within the injury site was normalized to a 100μm length of injury and subtracted from vehicle. On average ~1.3 sprouts were extended into the injury site by vehicle treated cultures, whereas Epo D-treated cultures produced ~3 and ~2.7 sprouts per 100μm of injury site when exposed to 0.1nM and 1nM of Epo D, respectively (Fig 4.4 E; $P<0.001$ and $p<0.05$, ANOVA, n=3 independent experiments). Despite the larger number of sprouts generated by Epo D-treated cultures, there was no change to average sprout length (Fig 4.4 F). Consistent with the finding that 100mM of Epo D affected microtubule stabilization, this same dose significantly reduced sprout outgrowth following 24 hours of treatment (Fig 4.4 D-E; ANOVA, $p<0.05$), as cultures extended only ~0.5 sprouts per 100 μm of the injury site. These results indicate that Epo D can modulate the post-injury sprouting response in a dose dependent manner.

As the previous western blot analysis had revealed that 100nM of Epo D could decrease Tau protein levels within 24 hours, it was assessed whether axonal sprouts, extending into the injury site, were in fact Tau immunolabeled. Results indicate that all axonal sprouts found after 24 hours of drug treatment with 100nM of Epo D are positive for Tau (data not shown).

To determine whether Epo D was promoting axon sprouting by excitatory projection neurons *in vitro*, the effect of Epo D treatment on post-injury axonal sprouting was studied in a specific subpopulation of principal or projection neurons. For this purpose, neurons expressing YFP under the control of the neuron-specific *Thy1* promoter were specifically examined. Neurons from *Thy1-YFP* transgenic mice were grown to relative maturity, and were injured using the complete transection method at 15 DIV. Cultures were treated with vehicle or Epo D for 24 hours. YFP-expression within the neurites allowed us to specifically detect YFP-positive spouts within the injury site (Fig 4.5 B). These sprouts were also Tau positive, indicating they were axons (Fig 4.5 A, C). Quantitative analysis of post-injury sprouting showed that 0.1nM Epo D significantly increased the number of YFP-positive sprouts elaborated into the injury site (0.58 ± 0.05 sprouts) compared to vehicle (0.28 ± 0.02 sprouts) (Fig 4.5 D). Epo D treatment did not affect mean sprout length of YFP-positive sprouts (Fig 4.5 E). These data indicate that the response of YFP^+ projection neurons to Epo D-treatment is consistent with the response of the total neuronal population represented by Tau-positive sprouts, demonstrating the Epo D can

broadly modulate the sprouting response of multiple neuron types.

4.3.5. Epo D does not influence the rate of post-injury sprout extension

Having established that Epo D could successfully increase the total number of sprouts elaborated, whether the drug was influencing the rate at which neurons extended these processes into the injury site was explored next. Two concentrations of Epo D (0.1nM and 100nM) based on their ability to significantly influence sprout outgrowth were examined (Fig 4.6). Cells were cultured in custom-made chambers for 15 days before transection injury. Immediately after injury cells were treated with vehicle or Epo D, and time-lapse movies were collected over the following 24 hours. As observed previously, neurites grew into the injury site under all conditions (Fig 4.6 A-C). The mean velocity of sprout outgrowth in vehicle-treated and Epo D-treated cultures was calculated, and found this to be comparable between treatments (Fig 4.6 D). It was also determined that Epo D treatment had no significant effect on the maximal speed of sprout outgrowth (Fig 4.6 E).

Since the previous results indicated that Epo D treatment had no significant effects on post-injury sprout length at 24 hours post-injury, it was next investigated if drug treatment could affect sprout length at different time points after injury. Using live imaging sprout length was examined after drug treatment during a 24 hour post-injury time period. As with previous results, sprout length was not significantly affected by drug treatment at any of the time points analyzed (Two-way ANOVA, $p>0.05$), although there was a trend towards reduction of sprout length with 100nM Epo D and increased sprout length with a low concentration of the drug of 0.1nM (Fig 4.6 F).

In summary these results show that moderate stabilization of microtubules can modulate the growth ability of neurons in culture.

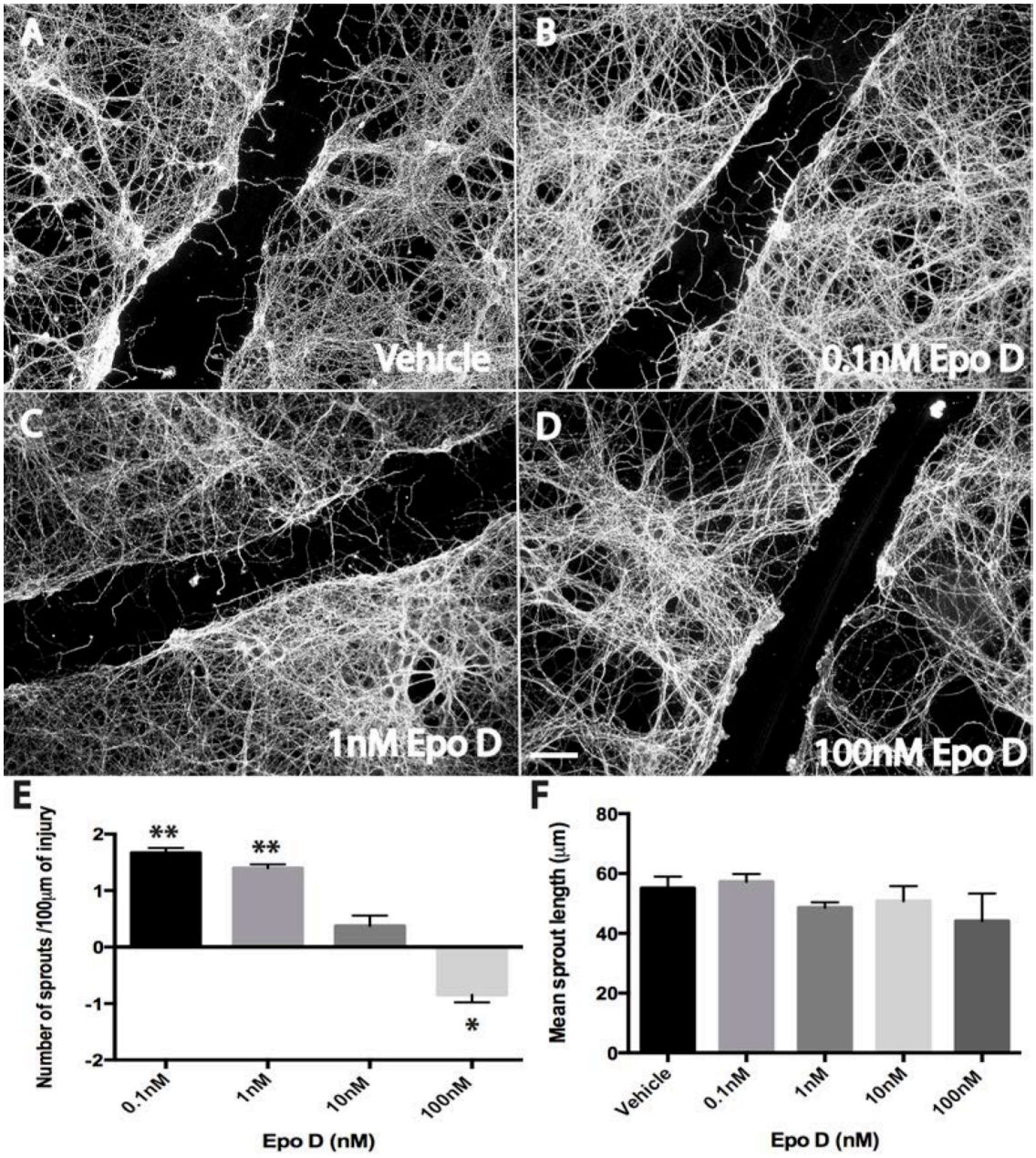


Figure 4.4. Tau immunofluorescence labeling demonstrating the effect of Epo D exposure on post-injury axonal sprouting at 15 DIV.

*24 hours post-injury the sprouting response was extensive and several sprouts had crossed the lesion site in vehicle treated cultures (A). Cultures treated with 0.1nM Epo D (B) and 1nM Epo D (C) showed an increased number of sprouts in the injury site, while cultures treated with 100nM Epo D (D) had a decreased number of post-injury sprouts relative to vehicle. Bar graphs represent the mean number of Tau positive sprouts per 100 μm of injury site length normalized to vehicle (E). Mean post-injury sprout length (F). Data are presented as mean ± SEM . One way ANOVA, followed by Bonferroni test *p<0.05, **p<0.01. Scale bar = 50 μm.*

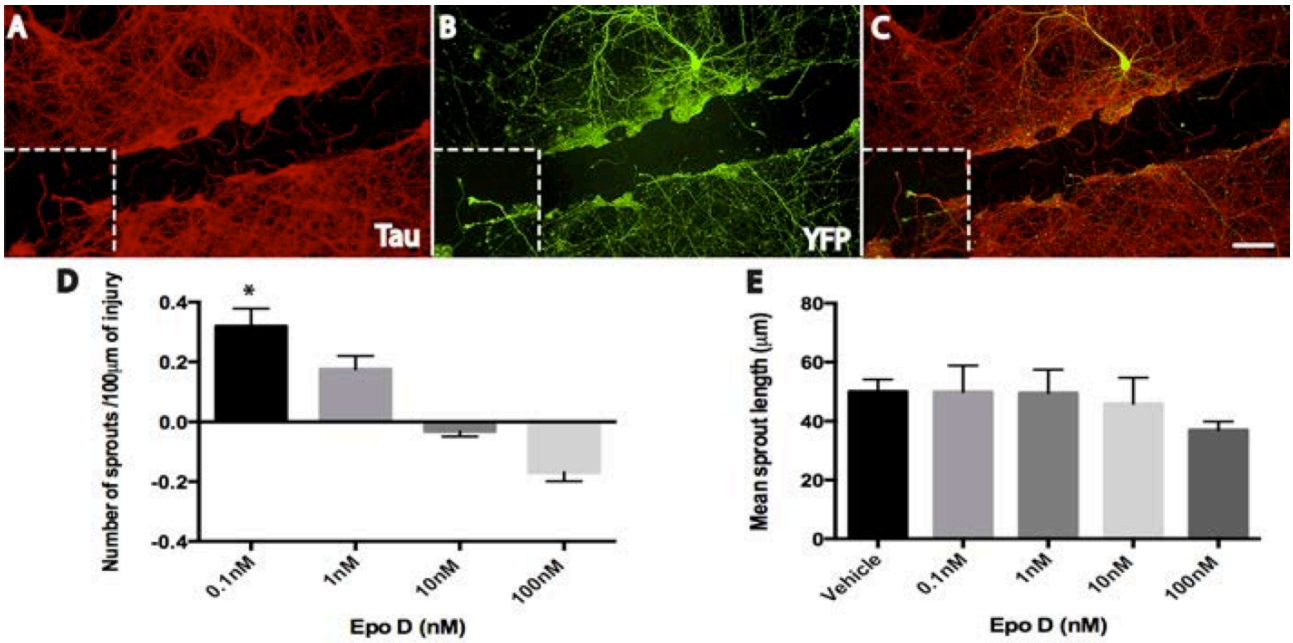


Figure 4.5 Number and mean length of Thy-1 YFP positive sprouts within the injury site after Epo D treatment.

*Tau (A) YFP labeling (B) of post-injury sprouts. Colocalization of Tau and YFP (C). Bar graph showing mean number of YFP positive sprouts per 100 μm of injury site length normalized to vehicle at 24 hours post-injury (D). Bar graph showing mean sprout length of YFP immunopositive sprouts at 24 hours post-injury and drug or vehicle treatment (E). Error bars are SEMs. One way ANOVA, followed by Bonferroni test *p<0.05. Data are presented as mean ± SEM. Scale bar = 40μm, for inset 20μm.*

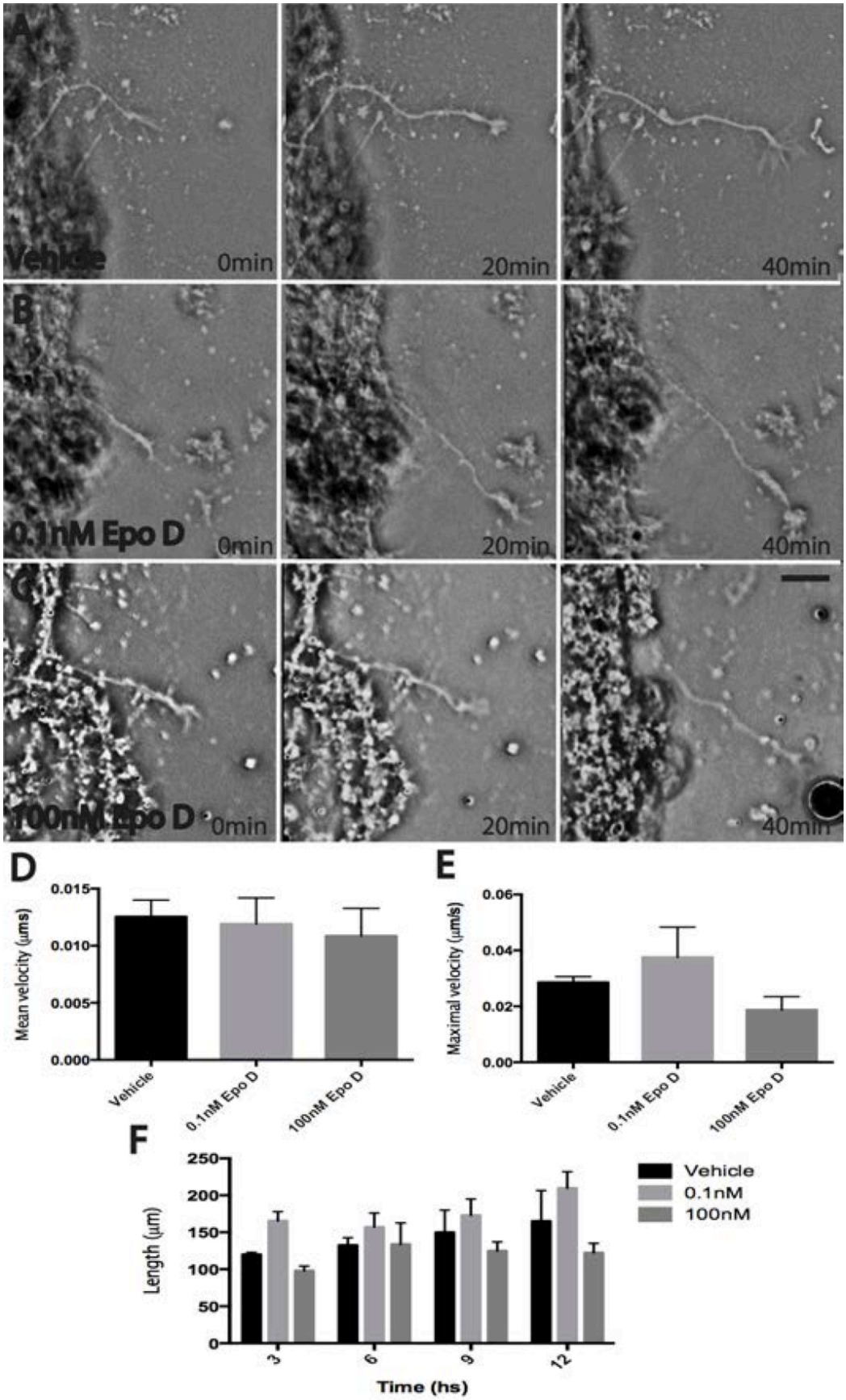

A
Vehicle
0min
20min
40min
B
0.1nM Epo D
0min
20min
40min
C
100nM Epo D
0min
20min
40min
D
Mean velocity (μms)
0.015
0.010
0.005
0.000
Vehicle
0.1nM Epo D
100nM Epo D
E
Maximal velocity (μm/s)
0.06
0.04
0.02
0.00
Vehicle
0.1nM Epo D
100nM Epo D
F
Length (μm)
250
200
150
100
50
0
3
6
9
12
Time (hs)
Vehicle
0.1nM
100nM

Figure 4.6. Post-injury sprout dynamics after Epo D treatment.

Live imaging of post-injury sprouts over a 40 minutes period of vehicle treated sprouts (A), 0.1nM Epo D treated sprouts (B), and 100nM Epo D treated sprouts (C). Bar graphs demonstrating the effect of Epo D treatment on post-injury spout growth velocity. Mean sprout growth velocity (D). Maximal sprout growth velocity (E). There were no significant differences in sprout growth velocity or maximal sprout growth velocity after drug treatment compared with control. Error bars are SEMs. One-way ANOVAa, followed by Bonferroni test. Time-lapse analysis of mean sprout length after drug treatment (F). There were no significant differences in sprout length at any of the time points analyzed after drug treatment. Two-way ANOVA multiple comparisons. Data are presented as mean ± SEM . Scale bar= 10 µm

4.4. DISCUSSION

Plasticity and regeneration of the mature CNS after trauma has been demonstrated in various models of injury such as stroke or focal and general structural brain injury (Chen et al., 2002, Carmichael, 2003, Blizzard et al., 2011, Canty et al., 2013). It has been well described that mature cells respond to injury with a substantial regenerative attempt indicated by the elaboration of sprout-like protuberances into the injury site (Chuckowree et al., 2004, Wieloch and Nikolich, 2006). To date, the beneficial outcomes of neuronal regeneration within the injury site *in vivo* are limited, as regenerative sprouts rarely result in functional recovery, and may result in aberrant non-functional axonal connectivity (McKinney et al., 1997, McKinney et al., 1999). Thus it is imperative to devise techniques targeted at manipulating the regenerative response of injured neurons. In this study, primary cortical neurons were grown to relative maturity and the effects of the microtubule-stabilizing drug Epo D on the post-injury axonal sprouting response were investigated. Using immuno-fluorescence-labeling and live imaging techniques, it was found that Epo D affects the number of sprouts elaborated into the injury site in a concentration dependent manner, without affecting the rate of growth of this sprouting response.

When studying cortical neurons *in vitro* it is extremely important to ensure that neurons model many key characteristics of the *in vivo* system. For the particular investigations presented here, it was of great importance to determine maturity of pyramidal neurons *in vitro* since previous investigations have demonstrated that the neuronal response to injury is dependent on neuronal maturity (Chuckowree and Vickers, 2003, Blizzard et al., 2007). Development of primary murine cortical neurons *in vitro* occurs via a sequence of steps across a defined time-course, involving neurite outgrowth, polarization and elongation followed by loss of immaturity markers such as growth cone markers and localization of synaptic proteins in dendritic spines as an indication of mature synapses (Dichter, 1978, Dotti et al., 1988, De Lima et al., 1997, Picken Bahrey and Moody, 2003). Neuronal development in culture in this study was assessed by the use of morphological, synaptic and electrophysiological characteristics (See Chapter 2). It was demonstrated that cortical neurons take approximately 2 weeks to mature to the stage where they form complex neuronal networks with multiple connections, have synaptophysin expression characteristic of functional synapses and fire action potentials (See Chapter 2). This developmental time-course of cortical neurons *in vitro* mirrors that of their *in vivo* development, where neurons in the cortical plate have been demonstrated to gain repetitive firing

ability by postnatal day 14 (Picken Bahrey and Moody, 2003).

This study determined the role that Epo D manipulation of microtubule stabilization plays in regeneration. Epo D has been shown *in vivo* to promote polymerization of tubulin heterodimers into microtubules and stabilize pre-formed microtubules, and has recently generated interest as a potential candidate for protection from neurodegeneration (Andrieux et al., 2006, Brunden et al., 2010, Brunden et al., 2011, Barten et al., 2012, Cartelli et al., 2013). However, Epo D is most commonly used as a drug in cancer therapy, (Wang et al., 2005a) where it exerts it action by binding to tubulin and causing a microtubule-mediated obstruction of mitosis and cell division, producing an arrest of cell growth or apoptosis (Cortes and Baselga, 2007, Cheng et al., 2008). The results show that Epo D had no significant effects on neuronal viability, metabolic function or cellular health when applied directly to neurons, at any of the concentrations applied. This indicates that cortical neurons are capable of tolerating up to 100nM of Epo D with no evident negative consequences on neuronal viability. Supporting the results presented here, previous research has shown that the use of Epo D in the treatment of neurodegenerative diseases requires a lower concentration of active drug for efficacy than the concentrations used for cancer therapy, and that no side effects are found in mice treated with systemic low doses of the drug (Beer et al., 2007, Brunden et al., 2010, Barten et al., 2012, Brunden et al., 2012, Fournet et al., 2012).

To determine the effect of Epo D on microtubule dynamics in cultured neurons, tubulin acetylation, and total Tau levels were investigated. Tubulin acetylation is a widely used marker of stable polymerized microtubules, with α-tubulin becoming acetylated at lysine-40 within stable microtubules, and conversely α-tubulin becoming rapidly deacetylated within non-polymerized microtubules (Conde and Caceres, 2009, Janke and Kneussel, 2010). This data indicates that relatively low doses of Epo D, such as 0.1nM, are sufficient to increase axonal sprouting, but are unable to alter tubulin acetylation within 90 minutes or 24 hours of application. In contrast results demonstrate that 100nM Epo D treatment, which significantly decreases the axonal sprouting response at 24 hours post treatment, causes a significant initial rise in acetylated tubulin that is resolved by 24 hours.

The effects of Epo D on total protein Tau level was also investigated. Tau is a microtubule-associated protein (MAP) that has been demonstrated to be an integral component of axon formation and neuronal transport, and can serve as a substrate for several cellular signaling pathways (Tytell et al., 1984). Results

presented in this Chapter indicate that 100nM of Epo D produces a significant decrease in Tau levels at 24 hours post treatment. Since Tau is known to stabilize microtubules, (Drechsel et al., 1992) these data suggest that prolonged high dose Epo D exposure effectively destabilizes microtubules, giving some indication that a prolonged exposure to a high concentration of the drug could produce negative consequences and needs further investigation. Similar findings were previously reported in Paclitaxel trials. It has been demonstrated that Paclitaxel can significantly reduce total Tau levels and Tau mRNA in a dose-dependent manner, and can also affect the amount of Tau incorporated into microtubules (Kar et al., 2003, Dickey et al., 2006). A relatively low dose of Epo D (0.1nM), on the other hand, did not significantly alter acetylated tubulin or Tau levels at either 90 minutes or 24 hours following application indicating that this dose of Epo D did not drive the stabilization of microtubules but rather promotes polymerization. Therefore, the results presented here provide compelling evidence to support the notion that Epo D targets microtubules and can increase microtubule stabilization (Barten et al., 2012).

To investigate drug effects on post-injury sprouting, mature cultures were injured using an established scratch model of injury. This *in vitro* model of complete transection injury is capable of eliciting substantial axonal sprouting at 24 hours post-injury. Whilst this injury is unable to replicate the complexity of the injured brain, it is a powerful tool for investigating drug effects on neuronal regeneration, screening for treatment targets and identifying new injury biomarkers (Chung et al., 2002, Chuckowree and Vickers, 2003, Loov et al., 2013). In this study Epo D was added to the culture media immediately after transection injury. The administration of a high concentration of Epo D (100 nM) resulted in a substantial reduction of the axonal sprouting response by approximately 50%, in respect to vehicle. This suggests that the stabilization of microtubules, evidenced by the initial increase in acetylated tubulin and loss of Tau reduces the number of axons that are able to respond to injury with the formation of axonal sprouts. Conversely, the administration of lower concentrations of the drug (0.1nM, 1nM) significantly increased the number of Tau immunolabeled sprouts directed into the injury site. These data indicate that Epo D promotes polymerization but not stabilization of microtubules, and increases the ability of neurons to regenerate.

Tau pathology in TBI has been demonstrated in animal models and in human studies (Ojo et al., 2013, Ojo et al., 2016, Puvenna et al., 2016). Given the drug's effects on tau demonstrated in this study and previous evidence indicating that there

are beneficial effects of epothilone treatment in a range of tauopathies, it is plausible that Epo D treatment in TBI might improve outcomes after injury not only by modulating axonal plasticity through microtubule stabilization but also by compensating for the possible loss of function of tau present after injury.

Previous studies in Chapter 2 have indicated that subtypes of CNS neurons respond differently to injury (Blizzard et al., 2011). Consistent with results from Tau immunolabeled axonal sprouts, 24 hours post-injury Epo D treatment on neurons derived from mice expressing YFP under the *Thy1* promoter pyramidal neurons resulted in a significant increase in the number of sprouts into the injury site. This implies that the microtubule manipulation of the post-injury response is important for subpopulations of pyramidal neurons.

No significant effect of Epo D on sprout outgrowth speed or mean sprout length was found. Previous studies indicate that Paclitaxel can promote axonal elongation in the injured optic nerve, and can increase elongation of dorsal root ganglion (DRG) neurons after spinal cord injury (Hellal et al., 2011, Sengottuvel et al., 2011). The data presented in this Chapter suggests that in an isolated system of cortical neurons, rate of outgrowth may not be reliant upon microtubule dynamics. However, it has been reported that the regenerative response of cut axons is correlated with the length of the remaining stump (Gomis-Ruth et al., 2008, Cengiz et al., 2012). Thus it is plausible that by using the scratch injury model where numerous axons are severed at once (added to the fact that the length of post-injury sprouts was only measured in the cell free scratch area), could have a confounding effect possibly masking a drug influence on post-injury sprout length and outgrowth speed.

The CNS response to injury is complex, involving a multifaceted sequence of events that plays out over a broad time course. Due to this complexity, it remains unclear whether regenerative sprouting is beneficial or detrimental (Cafferty et al., 2008b). Experimental models have demonstrated that neuronal regeneration and plasticity can lead to improvements in functional outcomes (Rossignol et al., 1999, Weidner et al., 2001, Bareyre et al., 2004). However other models suggest that post-injury sprouting may result in aberrant axonal connectivity, possibly contributing to the development of epilepsy post-injury (Prince et al., 2009, Hunt et al., 2013). This leads to the notion that axonal regeneration may be advantageous in one facet and then deleterious in another. In this regard, the opposing effects of low and high doses of Epo D on post-injury sprouting could be beneficial. A drug that could control

neuroplasticity in a dose dependent manner could be of great importance as a therapeutic intervention. Further studies would need to focus in elucidating the optimal timing of drug delivery to ensure treatment with different concentration of the drug are given are at the right time during the progression of the disease to ensure beneficial outcomes.

A further pathological characteristic of the neuronal response to injury is the formation of focal axonal swellings, known as axon bulbs, either at the end of a completely axotomised axon or as a swelling in an intact injured axon (Maxwell, 1995, Meythaler et al., 2001, Smith et al., 2013, Hanell et al., 2014). Such axonal bulbs, present in both grey matter and white matter tracts in the injured brain, are associated with a disruption in axonal transport, which is mediated by microtubules (Maxwell, 1995, Erturk et al., 2007, Tang-Schomer et al., 2012). Although not the focus of the current investigation, determining the effect of Epo D's manipulation of microtubules on the formation and persistence of axonal bulbs following injury is an intriguing avenue for future investigations.

The findings of the current study have important implications for the understanding of Epo D as a possible therapeutic intervention for TBI. Unlike Paclitaxel, Epo D can penetrate the BBB making it an ideal candidate as a potential therapeutic. The data presented here support cortical neurons being able to tolerate relative high concentrations of Epo D, and indicate that this drug can increase microtubule stabilization by increasing tubulin acetylation. The results show that at relative high doses Epo D can discourage neuronal sprouting, and at low doses it promotes polymerization of microtubules while maintaining dynamic function and encouraging neuronal growth. This data is consistent with Epo D regulating neuronal sprouting by stabilizing microtubules in a dose dependent manner and represent the critical first step in the pathway of investigating Epo D as a potential candidate for controlling axonal regeneration following injury. Collectively, these studies pave the way for future investigations targeting axonal regeneration in the mature central nervous system following trauma.

5. DISCUSSION

Traumatic brain injury (TBI) remains a global leading cause of death and disability in young people (Leibson et al., 2011, Reis et al., 2015). Moreover TBI has been associated with the development of chronic neurodegenerative disorders like Alzheimer's disease and Parkinson's disease (Moretti et al., 2012, Bigler, 2013, DeKosky et al., 2013, Esopenko and Levine, 2015, Faden and Loane, 2015, Li et al., 2016). Currently the treatment of TBI is limited to intensive clinical care immediately after injury and long-term rehabilitation (Diaz-Arrastia et al., 2014, Kochanek et al., 2015). However, there is a lack of clinically proven neuroprotective, neuroregenerative and therapeutic agents that can limit secondary injury and enhance repair. In fact in the last two decades more than 75 clinical trials have tested treatments for TBI, but none of the treatments studied has been proven to safely and effectively reverse the damage brought on by the injury (Yue et al., 2013, Diaz-Arrastia et al., 2014, Stein, 2015). This lack of clinical translation is directly related to an incomplete understanding of the mechanisms activated after injury. It is assumed that the vast and deleterious consequences of TBI are associated to the incapacity of the adult brain to completely repair after an insult. Therefore, identifying the cellular response to injury is vital for developing effective future therapies to treat brain injury. Moreover, elucidating cell-type specific pathologies would not only help consolidate our current understating of disease mechanisms but also potentially identify novel therapeutic targets.

There is evidence that suggests that the response to injury depends not only on the injury type but also on the neuronal cell type that might be affected. Thus this thesis utilized *in vitro* and *in vivo* methodological approaches, focusing on specific neuronal subpopulations within the neocortex, to provide novel insight into how the mature brain responds to injury. The central hypothesis of this thesis was that excitatory and inhibitory neurons respond differently to trauma. The mechanisms activated after injury were investigated with particular focus on axonal regenerative response, compensatory plasticity and their functional consequences. In order to address the hypothesis, this thesis investigated three aims. In Aim 1 axonal sprouting and dendritic remodeling were characterized following i*n vitro* axonal transection of primary cortical neurons, with responses compared between excitatory and inhibitory neuronal populations (Chapter 2). The functional consequence of this post-injury response was investigated using electrophysiological techniques. To extend upon the observations of Aim 1, and investigate the susceptibility of interneurons to injury i*n vivo*, as described in Aim 2 of this thesis, alterations in the inhibitory network and

in a specific subpopulation of interneurons were characterized following the induction of mild TBI using the lateral FPI model (Chapter 3). Additionally, the functional output of interneurons following FPI was investigated using electrophysiology. Finally, axonal sprouting manipulation through the administration of a microtubule-stabilizing drug, Epothilone D, was thoroughly investigated in Chapter 4 using an *in vitro* model of injury.

Axons are highly vulnerable to injury as they project over long-distances and have a complex and highly organized structure (Meythaler et al., 2001, Johnson et al., 2012). After injury, axons attempt regeneration in the form of a sprouting response (Chuckowree and Vickers, 2003, Blizzard et al., 2011, McKillop et al., 2016). This regeneration or reorganization after injury could lead to an adaptive reconnection to the original targets or to maladaptive processes in the form of aberrant connections that can be linked to epileptogenesis (Prince et al., 2009, Hunt et al., 2013). In the current thesis, *Thy-1 YFP* transgenic mice that express YFP in a subset of excitatory neurons were used to investigate cell type specific responses to injury. Data obtained from *in vitro* experiments show that mature pyramidal neurons attempt regeneration and sprout into the injury site indicating that the axonal regenerative response is an intrinsic response to injury that is neuron type-specific. The response of a population of *GAD67-GFP* positive inhibitory neurons was also investigated using immunocytochemical techniques. In this population, there were no axonal sprouts present in the injury site. Further results presented in Chapter 2 indicate that the initiation of the post-injury sprouting response in pyramidal neurons after transection injury is likely linked to an increased excitability, demonstrated by an increased firing frequency in these neurons. Post-injury hyperexcitability has been established in the past, whether it is a protective or pathogenic mechanism related to the development of post-traumatic epileptogenesis remains inconclusive and further studies at later post-injury time-points would be required to establish the long term consequences of the early excitability found.

Interestingly *in vitro* studies in Chapter 4 determined that the axonal sprouting response can be modulated by microtubule stabilization; high doses of the microtubule-stabilizing drug Epothilone D discouraged neuronal sprouting, while low doses promoted axonal growth (Brizuela et al., 2015). These findings indicate that Epothilone D may be a good candidate for manipulating axonal regeneration following injury in a dose-dependent manner, possibly preventing aberrant connections and hyperexcitability when administered at high doses, or stabilizing

damaged microtubules to promote axonal recovery following low dose exposure. Whether post-injury axonal sprouting could contribute to functional recovery after injury remains contentious (Schwab, 2002, Lee et al., 2014, Geoffroy et al., 2015). In this regard, the opposing effects of low and high doses of Epothilone D on post-injury sprouting could be beneficial, as encouraging axonal regeneration may be advantageous in one facet and then deleterious in another. Therefore a drug that could control neuroplasticity in a dose dependent manner could be an important therapeutic intervention after TBI. Further studies would be aimed to investigate the appropriate timing of treatment with Epothilone D to take advantage of the drug's concentration dependent effects on axonal sprouting. Moreover future studies would need to be carried out using *in vivo* animal models of injury in order to determine if treatment with this microtubule-stabilizing drug can promote brain healing and functional recovery following traumatic injury to the brain.

The neuronal response to injury is not limited to axonal regeneration. After a traumatic injury to the brain neurons can respond and attempt regeneration through the spontaneous rearrangement of the affected network through the induction of structural or morphological plasticity (Macias, 2008). This thesis demonstrated cell-type specific injury induced morphological changes using an *in vitro* model of injury. It was determined in Chapter 2 that only a specific population of interneurons, and not all subtypes of interneurons or pyramidal neurons, has the capacity for structural dendrite remodeling directed away from the injury site. Calretinin interneuron remodeling towards undamaged areas could be related to the loss of original connections and the formation of new synaptic connections. Electrophysiological studies in Chapter 2 point to a possible link between dendritic remodeling of inhibitory neurons, axonal sprouting of excitatory neurons and hyperexcitability after injury given that they where found at the same time point post-injury in the same injured area. Although as previously discussed in Chapter 2 it is also plausible that the different response to injury of excitatory and inhibitory neurons found *in vitro* is related to a differential effect of diffusible factors released around the injury onto these specific neuronal subtypes.

As previously stated, while maladaptive axonal sprouting of excitatory neurons has been previously linked to hyperexcitability (Prince and Tseng, 1993, Prince et al., 1997), it is plausible that decreased inhibitory input due to dendritic remodeling of interneurons towards undamaged areas also plays a role in the increased excitability found. Results in Chapter 3 further point to a decrease in

inhibitory input after injury related to alterations in the dendritic arbor of the same subpopulation of inhibitory neurons. This finding provides further powerful insight into the capacity of the CNS for compensatory plasticity and the change in the balance between excitation and inhibition that can develop as a consequence. Future studies would be aimed to investigate functional alterations in CR interneurons due to post-injury dendritic remodeling by performing patch clamp recordings from these neurons.

A majority of cases of TBI (approximately 70-80 percent) are mild injuries that involve only brief loss of consciousness (Arciniegas et al., 2005, Dewan et al., 2016). Although the deficits produced by mild TBI are frequently subtle, memory, affective and executive dysfunction can nevertheless emerge and cause substantial impairment and life disruption (Albrecht et al., 2016, Girgis et al., 2016). GABA is the main inhibitory neurotransmitter in the CNS and GABAergic interneurons play an important role in the synchronization of network activity. Therefore, alterations in the inhibitory circuit could lead to shifts in the balance between excitation and inhibition that may result in cognitive impairment (Almeida-Suhett et al., 2015, Drexel et al., 2015, Smith et al., 2015, Carron et al., 2016). Using an *in vivo* model of injury this thesis demonstrated that trauma induced cortical reorganization in the form of sub-population specific morphological changes of inhibitory neurons; a decrease in dendritic arbor complexity was found in Calretinin interneurons after injury. The vulnerability of this subpopulation of interneurons, in the form of loss or reorganization of dendrites, has been described in animal models of ischemia and epilepsy (Toth et al., 2010, Toth and Magloczky, 2014). Results presented in Chapter 3 also demonstrated cortical changes in inhibitory input onto excitatory neurons after mild TBI with no overall loss of interneurons. Calretinin interneurons are suggested to play a key role in the inhibitory network since they can effectively synchronize dendritic inhibitory interneurons. Therefore it is possible that the post-injury dendritic remodeling of this population of interneurons is related to a decreased inhibitory input onto pyramidal neurons that leads to an excitation-inhibition imbalance after mild TBI. Hence, targeting these inhibitory neurons after injury could potentially improve functional deficits found as a result of TBI. Pharmaceutical therapeutic agents that directly target the inhibitory circuit have been used successfully in other diseases where excitability and interneuron alterations are present (Rudolph and Mohler, 2014, Schousboe et al., 2014). However, in TBI this must be treated with caution as it has been demonstrated in this thesis that the response to injury is a complicated evolving time line of cell specific neuronal changes. Therefore, manipulation to either

induce axonal regeneration with microtubule stabilizing agents, as demonstrated in this thesis, or potentially targeting the inhibitory system, would need to be precisely timed. Future directions of the current thesis would be trialling these potential therapeutic interventions.

5.1. CONCLUSION

The adult brain retains the capacity to remodel and attempt to compensate for functional loss after injury, although it remains unclear whether all neuronal subpopulations have the same plastic ability. This thesis demonstrated that different subpopulations of neocortical interneurons respond to injury in a specific and unique manner different from other populations of interneurons and excitatory neurons. Furthermore, the findings of the thesis indicate that post-injury axonal sprouting of excitatory neurons and dendritic remodeling of inhibitory neurons result in potentially functionally inappropriate outcomes. However, it was also demonstrated that axonal sprouting could be modulated by microtubule stabilization. Moreover, it was established that injury induces morphological changes in a subpopulation of interneurons and that those changes can be related to functional abnormalities after injury. Overall this thesis presented insight into a differential response to injury of excitatory and inhibitory neurons that may provide novel therapeutic targets for the treatment of TBI.

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7. APPENDIX

7.1 General and Cell culture solutions

0.01M PBS, pH 7.4

100mL 9% NaCl (90g of NaCl (Sigma, USA) per 1L Milli-Q® water)

40mL Na2HPO4 (BDH) (28.4g per 1L Milli-Q® water)

10mL NaH2PO4.2H2O (Sigma) (31.2g per 1L Milli-Q® water)

850mL Milli-Q® water

0.001% poly-l-lysine

450mL sterile 0.01M PBS

50mL 0.01% poly-l-lysine (Sigma, USA)

7.2 Immunocytochemistry and immunohistochemistry solutions

0.3% Triton/PBS

600µL Triton X (Sigma)

200mL 0.01M PBS

4% Paraformaldehyde (PFA)

40g PFA (Sigma, USA)

40g Sucrose (Sigma, USA)

100mL 9% NaCl

400mL Na2HPO4

500mL NaH2PO4.2H2O

Heat while stirring in a fume hood.

7.3 Immunoblotting solutions

10% Sodium Dodecyl Sulfate (SDS)

10g SDS (BDH, USA)

Dissolve in 90mL of Milli-Q® water with gentle stirring and adjust to 100mL with Milli-Q®.

5X Running Buffer, pH 8.3

9g Tris base

43.2g Glycine (Bio-Rad, USA)

3g SDS

Combine and add 600mL of Milli-Q® water. Combine 100mL Running Buffer with 400mL Milli-Q® water prior to use

Transfer Buffer

3.03g Tris base

14.4g Glycine

200mL Methanol (Sigma, USA)

Make up to 1L with 800mL of Milli-Q® water.

Tris buffered saline (TBS)

4.84g Tris base

58.4g NaCl

1.5L Milli-Q®

Adjust to pH 7.5 with 10M HCl and make up to 2L with Milli-Q® water.

Tris buffered saline with Tween (TTBS)

1.1L TBS

550uL Tween-20 (Bio-Rad, USA)

TTBS/5% skim milk powder

100mL TTBS

5g skim milk powder

Store at 4°C for a limited time.

12% Separating gel

3.35mL Milli-Q® water

2.5mL 1.5M Tris-HCl, pH 8.8

100µL 10% SDS

4mL Bis/Acrylamide

Add quickly immediately prior to pouring

50µL 10% APS (Bio-Rad, USA)

5µL TEMED (Bio-Rad, USA)

Combine ingredients in the listed order and pour gel immediately. Allow to polymerization.

4% Stacking gel

6.1mL Milli-Q® water

2.5mL 0.5M Tris-HCl, pH 6.8

100µL 10% SDS

1.33mL Bis/Acrylamide

50µL 10% APS

10µL TEMED